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Mechanisms of resistance of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) to organophosphorous insecticides

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Mechanisms of resistance of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) to organophosphorous insecticides

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Resumen

La mosca Mediterránea de la fruta, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), es una de las plagas de mayor incidencia económica en el sector citrícola español. El manejo de esta plaga se basa en el uso de compuestos organofosforados (OPs), principalmente malatión. A pesar de que en España esta plaga se trata, con OPs, desde 1955 en las áreas citrícolas, hasta el momento no se han citado fallos en su control (Viñuela, 1998). Sin embargo, en la Comunidad Valenciana, el número de tratamientos insecticidas se ha incrementado en los últimos años, lo que podría incidir en un incremento de la tolerancia de *C. capitata* a malatión. En este contexto, se ha investigado la resistencia a malatión en poblaciones de campo sometidas a una elevada presión de selección, asimismo, se ha determinado la posible resistencia cruzada a otros insecticidas disponibles y se han establecido los principales mecanismos de resistencia.

Para determinar la susceptibilidad a malatión en poblaciones de campo, se recogió durante 2004 ó 2005 fruta infestada con larvas de *C. capitata* procedente de diferentes áreas geográficas: siete en la Comunidad Valenciana (Burriana, Castellón, Villareal, Carlet, Alcudia, Serra y Orihuela), una en Gerona (Bajo Ampurdán) y otra en La Rioja (Albeda). Todas las poblaciones de campo mostraron una menor susceptibilidad a malatión (6 a 201-veces) cuando se compararon con dos poblaciones de laboratorio (Lab-INIA y Lab-IVIA), encontrándose una relación entre la frecuencia de tratamientos recibidos y la resistencia a malatión. Poblaciones que recibieron el mayor número de tratamientos (5-10 tratamientos/año), mostraron los mayores niveles de resistencia (LC50 entre 1000 y 3000 ppm y LC90 mayor de 10000 ppm). Estos hechos son de gran relevancia para el control de esta plaga en la Comunidad Valenciana, ya que la concentración de malatión que se emplea en los tratamientos aéreos es de 7500 ppm.

Una línea resistente (W) procedente de la población recogida en Castellón, y una población susceptible de laboratorio, Lab-IVIA (C), fueron seleccionadas para posteriores estudios. Ensayos de toxicidad, realizados con otros dos insecticidas permitidos para el control de *C. capitata*, mostraron que la línea W era

aproximadamente 10 veces significativamente más tolerante a fentión que la línea C. Estos resultados podrían indicar que el mecanismo que confiere resistencia a malatión pudiera conferir resistencia a otros OPs (resistencia cruzada) o que, debido a que el fentión fue utilizado para el control de *C. capitata* durante los años 60s, 70s y 80s, dos mecanismos de resistencia se hubieran desarrollado independientemente (multiresistencia). En cuanto al spinosad, no se encontraron diferencias significativas entre ambas líneas.

Se ha obtenido la secuencia codante del gen de la AChE en *C. capitata* (*Ccace*) a partir de individuos de la línea susceptible Una mutación Gly328Ala (numeración de acuerdo con la numeración de *Torpedo*), próxima a uno de los residuos de la triada catalítica en la AChE, parece estar asociada con la resistencia de *C. capitata* a malatión. Esta mutación se encontró en homocigosis en adultos de la línea W cuya AChE mostraba una reducción en su actividad y una menor sensibilidad a ser inhibida por el malaoxon (fenotipo WR). Por el contrario, la mutación no se encontró en ninguno de los individuos de la línea susceptible (fenotipo C), y se encontraba en heterocigosis o no se encontraba en adultos de la línea W cuya AChE fue sensible al malaoxon y su actividad no estaba alterada (fenotipo WS). Esta mutación coincide con una mutación descrita en otras especies de Diptera, *Drosophila melanogaster* y *Musca domestica*, en las que estaba relacionada con una menor sensibilidad a malaoxon y, en el caso de *M. domestica,* se comprobó que afectaba a la afinidad de la enzima por el substrato (Walsh *et al.*, 2001, Menozii *et al.*, 2004).

El uso de sinergistas puso de manifiesto la posible implicación de las esterasas en la resistencia a malatión, ya que DEF, un inhibidor de esterasas, incrementó su toxicidad 8,0-veces frente a la línea resistente. Sin embargo, ambas líneas mostraron pequeñas diferencias en la actividad esterasa frente a diferentes substratos, lo que contrasta con las grandes diferencias encontradas en especies donde una sobre-expresión de esterasas es la responsable de la resistencia a insecticidas, como en el caso de *Myzus persicae* (Field y Devonshire, 1998; Field *et al*., 1999; Blackman *et al.*, 1999) y *Culex pipiens* (Gullemaud *et al.*, 1997). Mutaciones puntuales en las ali-esterasas que alteran la especificidad de estas enzima, han sido asociadas con resistencia específica a malatión (MCE). Los resultados obtenidos muestran una reducción significativa en la actividad esterasa usando MtB como substrato (1,6 veces) en la línea W, cuando se comparaba con la línea C. Sin embargo, la hidrólisis de malatión resultó muy baja en ambas líneas, al contrario de lo que ocurre en otras especies donde este mecanismo de resistencia ha sido demostrado (Whyard et al., 1994; Campbell et al., 1998).

Asimismo, el TPP, sinergista que se emplea para el diagnostico de este tipo de resistencia, sólo incrementó ligeramente la toxicidad del malatión (3,2- y 2,0-veces en las líneas W y C, respectivamente). Además, al comparar las secuencias codantes del gen de la αE7 (*Ccα E7*) en individuos de ambas líneas, no se encontraron ninguna de las dos mutaciones asociadas con resistencia a OPs en otras especies de moscas (Campbell *et al*., 1998a,b; Claudianos *et al.*, 1999; Taskin and Kence, 2004; Taskin *et al.*, 2004). No obstante, algunos individuos de la línea W presentaron cambios de aminoácidos en la secuencia de *Ccα E7*, que podrían estar relacionados con la disminución en la actividad esterasa.

Summary

The Mediterranean fruit fly (Medfly), *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is considered one of the most economically damaging pests in the Spanish agricultural sector. The control of this pest has been accomplished chiefly by organophosphorous insecticides, mainly malathion mixed with protein bait. So far, no resistant field populations of *C. capitata* to malathion have been reported yet, despite being the most widely used insecticide for the control of this pest. However, in the last years the frequency of the treatments has been increased in some areas of the Comunidad Valenciana, which may result in an increased tolerance to malathion. In this context, it has been investigated the resistance to malathion in field populations subjected to high selection pressure, moreover, it has been determined cross resistance to other insecticides related to the control of this pest and has been established the main resistance mechanisms.

To determine the susceptibility to malathion in field Medfly populations, larvae-containing infested fruits were collected in 2004 or 2005 from fruit orchards from different geographical areas: seven from the Comunidad Valenciana (Burriana, Castellón, Villareal, Carlet, Alcudia, Serra y Orihuela), one in Cataluña (Bajo Ampurdán) and other from la Rioja (Albeda). All these populations showed lower susceptibility to malathion (6 - 201-fold) when compared with laboratory populations. Moreover, differences in susceptibility are related with the frequency of field treatments. Populations from the Comunidad Valenciana, subjected to the highest selection pressure (5-10 aerial treatments/year), showed the highest levels of resistance (LC_{50} between 1000 y 3000 ppm and LC_{90} over 10000 ppm). This finding is of great relevance for the control of this pest, since the concentration of malathion, in the protein baits, used in aerial treatments in the Comunidad Valenciana is 7500 ppm.

A resistant strain (W), derived from a field population from Castellón, and a susceptible strain (C), from lab-IVIA, were selected for further studies. The toxicity of another two insecticides (fenthion and spinosad) related to the control of *C. capitata* were tested by ingestion. The W strain was about 10-fold significantly more tolerant to fenthion than the C strain, whereas no significant differences were found with respect

to spinosad. These results may indicate that the mechanism that confer resistance to malathion confers also moderate resistance to other OPs (cross-resistance) or, different mechanisms could have been selected independently to confer resistance to both insecticides (multi-resistance) because fenthion was the most widely used insecticide for Medfly control in Spain during the 60s, 70s and 80s.

It has been obtained the coding sequence of the AChE gene in *C. capitata* (*Ccace*) from individuals from the susceptible strain. A mutation Gly328Ala (*Torpedo* numbering) adjacent to the glutamate of the catalytic triad in the AChE, seems to be associated with resistance of *C. capitata* to malathion. This mutation was found in homozygosis in adults from the W strain whose AChE showed reduced activity and was less sensitive to inhibition by malaoxon (WR phenotype). On the other hand, the mutation was not found in any individual from the C strain (C phenotype), and it was found in heterozygosis or was not present in adults from the W strain whose AChE was sensible to malaoxon and its activity was not altered (phenotype WS). This mutation has been associated with resistance in populations of *D. melanogaster* and *Musca domestica,* in both cases it conferred less sensitivity to malaoxon, and in the case of *M. domestica,* this mutation led to a reduction in the affinity of the enzyme for the substrate.

Bioassays using synergists indicate that esterases may be involved in the resistance of *C. capitata* to malathion, since DEF, an esterase inhibitor, enhanced its toxicity (8-fold) in the resistant strain. However, small differences in the specific activity against different substrates were found when the strains were compared. These results contrast with the pronounced increases in esterase activity reported for those species where resistance is caused by over-expression mediated by gene amplification, as in *Myzus persicae*. Point mutations that alter the specificity of the enzyme have also been involved in specific resistance to malathion (MCE). The results obtained showed a significant reduction (1.6-fold) in the esterase activity against MtB in the resistant strain compared to the activity in the C strain. However, very low levels of *in vitro* metabolism of malathion were detected in both the C and W strains, which contrast with the increase in the MCE activity reported for those species where this type of resistance has been confirmed. Likewise, the synergist TPP only slight increased the toxicity of malathion (3,2- and 2.0-fold in the W and the C strains, respectively). Moreover, when the coding sequences of the α*E7* gene (*Cc*α*E7*) from individuals from both lines were compared, none of the mutations associated with the resistance to OPs in other species of higher Diptera were found. Albeit, some individuals from the W

strain presented some amino acid substitutions in the *CcαE7* sequence, that may be involved in the reduction of the ali-esterase activity.

Resumen amplio en español

INTRODUCCIÓN

La mosca Mediterránea de la fruta, *Ceratitis capiata* (Wiedemann), es una de las plagas más importante a nivel mundial, debido a su gran polifagia y elevada capacidad de adaptación. Ha sido citada como plaga en más de 300 clases de frutales y hortícolas (Liquido *et al.*, 1991). En nuestro país, ataca un cultivo de gran relevancia para sector agrario, los cítricos, del que España es el mayor productor en la Unión Europea y el primer país exportador a nivel mundial (Anónimo, 2004).

Esta plaga produce importantes pérdidas económicas tanto directas, debidas a una disminución en la calidad del fruto y de la producción, como indirectas, debidas a las estrictas medidas de cuarentena en pre- y postcosecha que imponen los países importadores en los que esta plaga no está presente. Su control se basa principalmente, en el uso de insecticidas organofosforados (OPs). Actualmente, en la Comunidad Valenciana, una de las principales áreas citrícola, se lleva a cabo un plan de control basado en una red de monitorización y tratamientos aéreos y/o terrestres con malatión.

Uno de los principales problemas ligados al uso de insecticidas, es el desarrollo de resistencia. Actualmente, más de 500 especies son resistentes a al menos un tipo de insecticida (Hart and Pimentel, 2002). Hasta el momento y a pesar de que en España, esta plaga se trata con OPs desde 1955 en las áreas citrícolas (campañas de interés estatal, publicadas anualmente en el boletín oficial del estado), no se han citado fallos en su control (Viñuela, 1998). Sin embargo, en la Comunidad Valenciana, el número de tratamientos insecticidas se ha incrementado en los últimos años, lo que podría interpretase con un incremento de la tolerancia de *C. capitata* a malatión. Asimismo, el potencial de esta plaga a desarrollar resistencia a malatión ha sido demostrado en una línea de laboratorio (Koren et al., 1984).

Los insecticidas organofosforados son esencialmente inhibidores de las esterasas incluyendo las colinesterasas, siendo su molécula diana la acetilcolinesterasa (AChE,

EC 3.1.1.1) (Eto, 1974). Los OPs inhiben la AChE bloqueando su función principal, hidrolizar el neurotransmisor acetilcolina. La acumulación de este neurotrasmisor en el espacio intersináptico evita la repolarización de la célula nerviosa, produciendo finalmente la muerte del organismo (Eto, 1974). La mayoría de los organofosforados se producen en su forma inactiva, forma tion (proinsecticidas) y necesitan ser activados, forma oxon. Esta activación viene mediada por las citocromos P450 monooxigenasas. La forma oxon inhibe irreversiblemente la AChE, ya que se forma un compuesto covalente mas estable y de hidrolisis más lenta, que el complejo acil-enzima que se forma con la acetilcolina. El malatión, principal compuesto para el control de *C. capitata*, es un insecticida de amplio espectro y baja toxicidad para mamíferos. La molécula contiene, además del fosfotrieseter característico de los OPs, dos enlaces carboxilester cuya hidrólisis conduce a la detoxificación del insecticida. Su selectividad se debe a la elevada actividad carboxilestererasa en mamíferos, en contraste con su baja actividad en insectos susceptibles.

La resistencia a OPs, mediada por una modificación de la molécula diana, se ha descrito en muchas especies de insectos (Fournier and Mutero, 1994). Estas modificaciones pueden ser de tipo cuantitativo o cualitativo. La resistencia debida a una sobre-expresión del gen se ha mostrado en líneas resistentes de *Drosophila melanogaster* y *Aonidiella aurantii* (Levitin and Cohen, 1998; Charpentier and Fournier, 2001). Sin embargo, mutaciones puntuales en la AChE (23 identificadas hasta la fecha) que hacen que esta sea menos sensible a ser inhibida por el insecticida, es el principal mecanismo responsable de la resistencia a OPs (Mutero *et al.*, 1994; Zhu *et al.*, 1996; Vontas *et al*., 2001; Hsu *et al.*, 2006).

Asimismo, la resistencia a malatión puede ser debida a un incremento de la actividad de los principales sistemas metabólicos: citocromos P450 monoosigenasas (Welling *et al.*, 1974; Morton and Holwerda, 1985), glutatión S-transferasas (GST) (Wool *et al.*, 1982) y esterasas (Campbell *et al.*, 1998a; Karunaratne and Hemingway, 2001). Resistencia mediada por P450 o GST, generalmente supone un incremento en la cantidad de enzima. La resistencia mediada por esterasas, puede deberse a cambios cuantitativos o cualitativos. En muchos insectos, le resistencia a OPs es debida a un incremento en la actividad esterasa mediada por una amplificación de genes. Las esterasas secuestran y lentamente hidrolizan el insecticida, previniendo la inhibición de la AChE (Hemingway and Karunaratne, 1998). La resistencia específica a malatión se debe principalmente a carboxilesterasas que son capaces de hidrolizar los esteres específicos de malatión (MCE) (Hughes *et al.*, 1984; Hemingway, 1985). Este

incremento en la actividad MCE esta asociado con una disminución en la actividad esterasa general en especies de dípteros, tales como *Musca domestica* (van Asperen and Oppennorth, 1959), *Lucilia cuprina* (Hughes y Raftos, 1985) y *Chysomia putoria* (Townsend and Busvine, 1969). Este hecho ha sido explicado por la hipótesis "mutación en la ali-esterasa". Esta hipótesis propone que un cambio estructural en la esterasa *αE7* resulta en un cambio en la especificad de esta enzima, mostrando una reducción en la capacidad de hidrolizar substratos carboxilester y una capacidad para hidrolizar OPs. Dos mutaciones en esta enzima, Gly137Asp (numeración según *Drosophila*), que confiere resistencia a un amplio espectro de OPs y Trp251Ser/Leu (numeración según *Drosophila*), que confiere una resistencia específica a malatión, han sido descritas en *M. domestica* y *L. cuprina* (Campbell *et al.* 1998a; Taskin y Kence, 2004; Taskin *et al.*, 2004).

La participación de estos tres sistemas enzimáticos en la resistencia al insecticida puede ser deducido por el uso de inhibidores específicos denominados sinergistas. Estos compuestos bloquean e interfieren en la detoxificación del insecticida. Los mas comunes son: el piperonil butoxido (PBO) inhibidor de las P450 (Feyereisen, 2005); el dimetil maleato (DEM) inhibidor de las GST (Mulder and Ouwerkerk-Mahadevan, 1997); y el S,S,S-tributil fosforotioato (DEF) y el trifenil fosfato (TPP) como inhibidores de las esterasas (Oakeshott *et al.*, 2005).

OBJETIVOS

La presión de selección que se está ejerciendo sobre *C. capitata* con malatión en los últimos años en la Comunidad Valenciana puede haber resultado en la aparición de resistencia. Por ello, es necesario conocer la situación de susceptibilidad actual de las poblaciones de campo de *C. capitata* a malatión, estudiar los potenciales mecanismos de resistencia a OPs y determinar la posible resistencia cruzada a otros insecticidas permitidos para el control de esta especie. Esto ayudará al diseño de estrategias de manejo, para prevenir y minimizar el desarrollo, y evolución de la resistencia.

Los objetivos de este trabajo se indican a continuación:

1. Evaluar de la susceptibilidad a malatión de poblaciones españolas de *C. capitata* sometidas a distinta presión de selección en los últimos años. Asimismo se estudiaran la resistencia cruzada a otros insecticidas permitidos para su control y el efecto de los sinergistas.

2. Establecer el papel de la AChE, molécula diana de los OPs, como posible mecanismo de resistencia a malatión. Se secuenciará el gen de la AChE y se estudiarán las propiedades bioquímicas y la sensibilidad a OPs de esta enzima en dos líneas, una resistente y otra sensible.

3. Determinar las bases bioquímicas y moleculares de la resistencia metabólica a malatión. Se estudiará el potencial de detoxificación y el papel de la ali-esterasa en la resistencia de *C. capitata* a malatión.

RESULTADOS

Resistencia a malatión en poblaciones de campo de *C. capitata*

Para evaluar la susceptibilidad a malatión de poblaciones españolas de *C. capitata*, se recogieron durante 2004 ó 2005 poblaciones de *C. capitata* de diferentes áreas geográficas. Siete en la Comunidad Valenciana, principal comunidad en la producción de cítricos (Burriana, Castellón, Villareal, Carlet, Alcudia, Serra y Orihuela), una en Gerona (Bajo Ampurdán) y otra en La Rioja (Albeda). Los detalles de tratamientos, frutos y año de recogida se recogen en la tabla 1. En cada uno de los campos se recogió fruta que contenía larvas de *C. capitata* y se llevó al laboratorio. Los adultos, procedentes de la fruta infectada, se utilizaron para realizar los bioensayos de toxicidad a malatión por ingestión. Dos poblaciones de laboratorio, una procedente del Instituto Nacional de Investigaciones agrarias (Lab-INIA) y otra procedente del Instituto Valenciano de Investigaciones Agrarias (Lab-INIA), fueron utilizadas como controles. Los resultados de estos ensayos se recogen en la tabla 2.

Table 1. Tratamientos, huéspedes y lugares donde las poblaciones de *C. capitata* fueron recogidas.

Área	Población	Año	Huésped	Tratamientos con malatión contra *C. capitata*
Comunidad Valenciana	Burriana (Castellón)	2004	Nispero	8-9 a.t./año en 2003 y 2004
	Castellón (Castellón)	2004	Cítricos	8-9 a.t. y 2-4 t.t./año en 2003 y 2004
	Villareal (Castellón)	2005	Kaqui	zona urbana, no tratado en los últimos 2-3 años
	Carlet (Valencia)	2004	Melocotón	8 a.t. en 2002, 6 a.t. en 2003 y 6 a.t. en 2004[b]
	Alcudia (Valencia)	2004	Melocotón	8 a.t. en 2002, 6 a.t. en 2003 y 6 a.t. en 2004[b]
	Serra (Valencia)	2005	Cítricos	no tratada en 2001, 2002, 2003 y 2004, 1 t.t. en 2005
	Orihuela (Alicante)	2005	Cítricos	9 a.t. y 4 t.t. en 2005
Cataluña	Bajo Ampurdan (Gerona)	2004	Manzana	1 t.t. en 2004
La Rioja	Albelda	2004	Manzana	campo no tratado proximo a otros campos frutales tratados

[a] t.a., tratamiento aéreo, t.t., tratamiento terrestre.
[b] 4-6 tratamientos adicionales con fentión.

La susceptibilidad a malatión se determinó en adultos de 3 a 5 días de edad mediante bioensayos por ingestión. El insecticida (Malfin EC50) era suministrado en la dieta y la duración del bioensayo fue de 48 horas (Tabla 2). Todas la poblaciones de campo ensayadas mostraron una menor susceptibilidad (LC_{50}>100 ppm) a malation que las dos poblaciones de laboratorio Lab-IVIA (LC50=16) y Lab-INIA (LC50=12 ppm) (Tabla 2). Asimismo parece existir una correlación entre el número de tratamientos y la susceptibilidad a malatión. Las poblaciones de la Comunidad Valenciana, recogidas en campos sometidas a un mayor número de tratamientos (5-10), mostraron la menor susceptibilidad al insecticida (LC_{50} entre 1000 y 3000 ppm), con una razón de la concentración letal (RCL), con respecto a la población Lab-IVIA, en el rango de 66 a 201. Las poblaciones recogidas de campos que habían recibido un tratamiento, Bajo Ampurdán y Serra, mostraron una susceptibilidad intermedia (LC50 entre 500 y 1000 ppm), con una RCL entre los valores 33 y 60. Y las poblaciones recogidas de campos que no habían recibido ningún tratamiento, Villareal y Albeda, mostraron la mayor susceptibilidad al insecticida (LC50 entre 100 y 500 ppm) y unos valores de LCR de 6 y 30, respectivamente.

Table 2. Susceptibilidad a malatión de poblaciones de campo y laboratorio de *C. capitata*.

Tratamientos de campo con malatión	n	Pendiente ± E.S.	X^2	g.l.	CL_{50}[a] (95%LC)	RCL (CL_{50})[b] (95% LC)	CL90[a] (95% LC)
-	641	2,7 ± 0,2	80,5	19	16 (9 -20)	1	57 (42-110)
-	367	2,9 ± 0,3	29,8	18	12 (10-14)	0,8 (0,6 - 1)	36 (28-52)
5-10 /año	185	0,8 ± 0,1	27,1	18	1376 (668-3126)	88 (51 - 154)*	43965 (12468-894730)
5-10 /año	148	1,7 ± 0,3	7,4	18	3137 (2182-4850)	201 (133 - 305)*	18480 (10219-51674)
5-10 /año	136	0,8 ± 0,1	8,5	16	1636 (763-3629)	105 (49 -223)*	61271 (18699-655370)
5-10 /año	95	1,2 ± 0,2	14,5	13	1029 (503-2074)	66 (37 - 118)*	12232 (4897-92164)
5-10 /año	208	0,9 ± 0,1	20,5	22	1874 (1190-3300)	120 (72 - 202)*	41758 (16444-215180)
1 /año	97	0,8 ± 0,2	23	23	519 (130-1169)	33 (14 - 79)*	21039 (5796-1310400)
1/año	95	0,9 ± 0,2	15,2	10	938 (278-2735)	60 (30 - 122)*	19284 (5311-954910)
no tratada	251	1,6 ± 0,2	47,7	28	102 (44-160)	6 (4 - 10)*	639 (407-1476)
no tratada	66	1,2 ± 0,3	10,5	10	472 (163-1084)	30 (15 - 60)*	5739 (2021-182320)

[a] Malafin EC50 fue usado como insecticida. La concentración están expresadas en ppm de malatión en dieta.

[b] Ratio de la concentración letal (LCR) a la LC50 de cada población respecto a la población de laboratorio lab-IVIA.

[*] Las concentraciones letales (CL_{50}) son significativamente diferentes ($P<0,05$) si los límites fiducaiales al 95% no incluyen el valor 1 (Robertson y Preisler, 1992).

De acuerdo con los resultados de susceptibilidad a malatión, dos líneas, una de laboratorio Lab-IVIA (C) y otra resistente (W) procedente de la población de campo recogida en Castellón, fueron mantenidas en el laboratorio para posteriores estudios.

Se realizaron bioensayos para determinar la toxicidad del malatión por vía tópica y por ingestión (Tabla 3). En el bioensayo por aplicación tópica, el insecticida Malafin EC50 fue diluido en acetona, para obtener un rango de dosis, y una gota de 0,5 µl fue aplicada, mediante un microaplicador manual, sobre la parte dorsal del tórax de los adultos que, previamente, habían sido inmovilizados a baja temperatura (30 min a 4ºC). Los resultados muestran, independientemente de cómo se aplicó el insecticida, en la dieta o por vía tópica, diferencias significativas entre las dos líneas ensayadas (C y W). En ambos casos, W fue más resistente a malatión que C, aunque las diferencias fueron mayores cuando el insecticida fue suministrado en la dieta.

Otros insecticidas aprobados en España para el control de *C. capitata* en cítricos son: el fentión y el spinosad. El fentión, insecticida organofosforado, fue muy utilizado durante los años 60s, 70s y 80s para el control de *C. capitata* (Lloréis-Climent y Gilbert-Artiges, 1997), pero actualmente su uso es muy limitado y no está permitido para frutos destinados a la exportación. El spinosad es un compuesto de origen

natural, que ha mostrado ser efectivo para el control de *C. capitata* (Adan *et al.*, 1996), aunque todavía, su uso es muy límitado. La susceptibilidad de las líneas, C y W, a estos compuestos se ha determinado por ingestión. En ambos casos, los insecticidas, lebaycid (fentión) y el Spintor cebo (spinosad), fueron mezclados con la dieta para conseguir un rango de concentraciones. Ambos insecticidas resultaron más tóxicos para las dos líneas, C y W, que malatión. La línea W (LC_{50}=12 ppm) fue 9 veces significativamente más tolerante a fentión que la línea C (LC_{50}=1 ppm), mientras que no se encontraron diferencias significativas respecto al spinosad (LC_{50}=0.4 ppm para la línea C y 0.7 ppm para W).

Table 3. Susceptibilidad de las líneas de *C. capitata* susceptible C y resistente W a malatión, fentión y spinosad.

Insecticida	Ensayo	Línea[a]	n	pendiente ± E.S.	χ2	g.l.	CL_{50}[b] (95%LC)	LCR (CL_{50})[c] (95%LC)
Malatión								
	Ingestión	C	162	2,3 ± 0,4	16,7	16	18 (11-23)	1
		W	210	0,9 ± 0,1	62,1	32	1406 (664-3150)	79 (44-141)*
	Aplicación tópica	C	153	1,3 ± 0,2	24,4	15	1,2 (0,6-2,1)	1
		W	159	6,4 ± 1,4	39,5	17	27 (18-33)	22 (14-32)*
Fentión								
	Ingestión	C	108	1,8 ± 0,3	18,6	14	1,3 (0,6-2,2)	1
		W	144	2,6 ± 0,4	15,5	19	12 (10-15)	9 (6-16)*
Spinosad								
	Ingestión	C	278	2,3 ± 0,1	13,7	10	0,4 (0,3-0,5)	1
		W	164	1,9 ± 0,4	27,1	18	0,7 (0,4-0,9)	2 (1-3)

[a] El análisis probit fue realizado con las líneas C y W.

[b] Las concentraciones están expresadas en ppm de malatión, fentión o spinosad en la dieta para los ensayos por ingestión y como µg de malatión/peso vivo para los ensayos por aplicación tópica.

[c] Ratio de la concentración letal (RCL) a la CL_{50}, de la línea resistente respecto a la línea susceptible.

[*] los ratios de la concentración letal (RCL) son significativamente diferentes ($P<0.05$) si los límites fiducaiales al 95% no incluyen el valor 1 (Robertson y Preisler, 1992).

En una primera aproximación para determinar si la resistencia estaba mediada por una modificación de la molécula diana. Se midió la actividad específica de la AChE, en ausencia y en presencia de malatión (Malafin EC50), en extractos de cabezas de individuos pertenecientes a cada una de las dos líneas (C y W), utilizando el método

descrito por Ellman, (1961). La actividad específica de la AChE (nanomoles de acetilcolina hidrolizada/min/mg de proteína) de la línea W (61,4±2,1) resultó menor que la de la línea C (80,2±2,1). Además, la línea C mostró una mayor inhibición de la AChE que la línea W en presencia de concentraciones crecientes del insecticida (Figura 1).

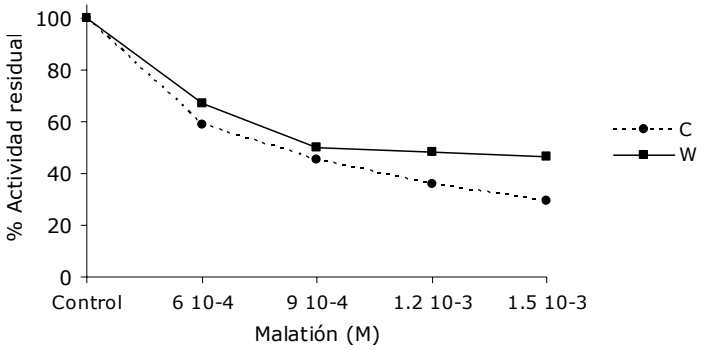

Figure 1. Inhibición *in vitro* de la actividad AChE por diferentes concentraciones malatión en las líneas C y W de *C. capitata*. Extracto de cabeza de 20 individuos de cada línea, fueron homogeneizadas y se midió la actividad residual de la acetilcolinesterasa a diferentes concentraciones de malatión.

Adultos de ambas líneas, C y W, fueron pretratados con sinergistas, por vía tópica 2 horas antes de suministrarles el malatión por medio de la dieta, para inhibir los principales sistemas de detoxificación (P450, glutatión S-transferasas y esterasas), y determinar su posible implicación en la disminución de susceptibilidad a malatión en la línea W (Tabla 4). Los sinergistas fueron: TPP (trifenil fosfato) y DEF (S,S,S-tributil fosforotritioate) como inhibidores de esterasas; PBO (piperonil butoxido) como inhibidor de P450; y DEM (dimetil maleate) como inhibidor de las glutation S-transferasas. En la línea W, DEF (8,0-veces) y TPP (3,2-veces) incrementaron significativamente el efecto del malatión, mientras que no se encontraron diferencias significativas en cuanto al DEM, y el PBO tuvo un efecto antagonista. En la línea C, ni PBO ni DEF tuvieron un efecto sobre la toxicidad del malatión, DEM tuvo un efecto antagónico y el TPP tuvo un efecto moderadamente sinérgico.

Table 4. Efecto de los sinergistas sobre la toxicidad de malatión en las líneas C y W de *C. capitata.*

Línea	Insecticida	Sinergista	n	Pendiente ± E.S.	χ^2	g.l.	CL_{50}[a] (95% LC)	Potencia[b] (95% LC)
C	malatión		319	2,4 ± 0,3	49,7	31	15 (11-19)	1
		+ TPP	148	2,1 ± 0,4	24,8	13	6,8 (2-11)	2 (1,3-3,4)*
		+ DEF	59	5,0 ± 1,6	1	4	16 (9-22)	1,14 (0,6-2)
		+ PBO	309	2,0 ± 0,3	97	30	18 (8-29)	0,8 (0,5-1,2)
		+ DEM	117	2,8 ± 0,5	7	10	25(19-33)	0,6 (0,4-0,9)*
W	malatión		568	0,9 ± 0,1	77	69	1303 (909-1881)	1
		+ TPP	133	0,9 ± 0,2	17,5	13	407 (177-1038)	3,2 (1,5-6,8)*
		+ DEF	108	0,8 ± 0,2	8,8	10	149 (25-407)	8 (3-21)*
		+ PBO	107	0,7 ± 0,2	20,6	12	5022 (1072 - 1037280)	0,3 (0,1-0,8)*
		+ DEM	49	0,8 ± 0,2	0,7	4	3491 (923 - 206410)	0,5 (0,1-1,6)

[a] Concentraciones en ppm de malatión.

[b] Potencia a la CL50 de la línea pretratada con sinergista con respecto a la misma línea tratada solamente con malatión.

[c] Las potencias son significativamente diferentes ($P<0.05$), si los límites fiducaiales al 95% no incluyen el valor 1 (Robertson y Preisler, 1992).

Resistencia a malatión mediada por modificación de la molécula diana

La actividad AChE se midió en extractos de cabeza de individuos de ambas líneas, C y W, por el método descrito por Ellman *et al.* (1961). Los individuos de la línea C presentaron una actividad específica de AChE dentro del rango de 60 a 190 nanomoles/min/mg de proteína. Sin embargo, la actividad AChE de los individuos de la línea W presentó una distribución bimodal, presentando dos tipos de individuos: individuos, con un rango de actividad semejante al de los individuos de la línea C, e individuos con una actividad menor, en el rango de 30 a 50 nanomoles/min/mg de proteína (Figura 2).

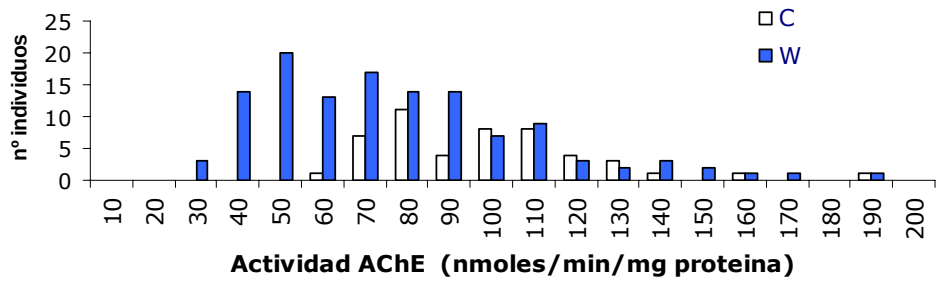

Figure 2. Actividad específica de la AChE en individuos de la línea C y W de *C. capitata*.

La sensibilidad de la AChE a ser inhibida por malaoxon fue ensayada en todos estos individuos. Los extractos de cabeza se incubaron durante 5 minutos con un aconcentracion 10^{-7} M de malaoxon antes de comenzar la reacción. Esta concentración produjo una inhibición del 50% de la actividad AChE en individuos de la línea C y en aquellos individuos de la línea W que presentaban una actividad AChE superior a 60 nanomoles/min/mg. Sin embargo, no produjo inhibición en aquellos individuos de línea W con una actividad menor a 60 nanomoles/min/mg.

Se difieren por tanto tres fenotipos: i) individuos de la línea W con baja actividad AChE y no inhibición por malaoxon (WR); ii) individuos de la línea W con actividad AChE normal e inhibición por malaoxon (WS); e iii) individuos de la línea C cuya actividad AChE era inhibida por malaoxon. La actividad AChE y los parámetros cinéticos se calcularon para cada uno de estos tres fenotipos (Tabla 5). La actividad AChE fue mayor en los fenotipos C y WS que en el WR. Asimismo, los individuos de fenotipo WR, mostraron un incremento en la k_m y una reducción en la V_{max}.

Table 5. Parámetros cinéticos de la AChE de los fenotipos C, WS y WR de *C. capitata*.

Fenotipos[a]	Actividad AChE ± E.S.[b] nmoles/ min/ mg	V_{max} ± E.S. nmoles/ min/ mg	K_m ± E.S. µM
C	79,7 ± 1,0	62,2 ± 1,2	77,8 ± 0,6
WS	91,3 ±2,1	71,9 ± 0,5	77,9 ± 3,4
WR	45,7 ± 0,8	34,4 ± 0,0	105,8 ± 1,4

[a] Para cada muestra se homogeneizaron las cabezas 30 individuos de cada fenotipo.
[b] Actividad AChE usando acetilcolina yodada como substrato. Los resultados se muestran como la media de tres réplicas ± el error estandar.

La posible alteración de la AChE, resultando en una reducción en la sensibilidad a la inhibición por OPs, fue investigada determinando la constante bimolecular de inhibición (ki) en los tres fenotipos C, WS y WR con respecto a diferentes OPs en su forma activada: malaoxon, azinfos-metil-oxon, paraoxon y clorpirifosoxon (Tabla 6). La AChE de los individuos WR mostró una menor sensibilidad a ser inhibida por malaoxon (ki, 0,05 10^7 $M^{-1}min^{-1}$) que los individuos, C y WS (0,1 y 0.1 10^7 $M^{-1}min^{-1}$, respectivamente). Sin embargo no se encontraron diferencias significativas entre ambas líneas respecto a su susceptibilidad a los otros tres insecticidas ensayados.

Table 6. Inhibición de la AChE en los fenotipos C, WS y WR de *C. capitata* por las formas oxon de compuestos organofosforados.

Fenotipos[a]	ki[b] (10^7 $M^{-1}min^{-1}$ ± E.S.)			
	Malaoxon	Azinfos-metil-oxon	Paraoxon	Clorpirifosoxon
C	0,11 ± 0,02	6,7 ± 1,7	0,09 ± 0,01	76,6 ± 5,6
WS	0,12 ± 0,04	5,7 ± 2,2	0,09 ± 0,01	67,5 ± 4,8
WR	0,05 ± 0,01	5,7 ± 2,1	0,09 ± 0,01	58,33 ± 8,7

[a] Para cada muestra se homogeneizaron las cabezas de 30 individuos de cada fenotipo.
[b] Constante bimolecular de inhibición. Actividad AChE usando acetilcolina yodada como substrato. Los resultados se muestran como la media de tres réplicas ± el error estandar.

Se ha obtenido la secuencia codificante del gen AChE (*Ccace*) a partir de individuos de la línea susceptible. Un primer fragmento se secuenció a partir de cebadores degenerados, diseñados en función de un fragmento muy conservado en la estructura

primaria de las AChEs. A partir de este fragmento se diseñaron cebadores específicos para la amplificación de los extremos 3’ y 5’ (Figura 3). La proteína muestras las principales características de las AChEs.

```
                                GTCGAGCTCCGCCAGCCATTCCGTAGTCAATCAGCTGAGTTTATTATTAAACAG     -150
TACATACAGCTCAGCCAATTATCAGCGAGTGTTTGCTAAATAAAACCTTTGCTGCGCTTTATTACAACAAATAT      -75
CCCAACCTACTCCTACCAACTAACCACCGTATCACCTACCACCCACTTCGACGAGCACACTCAATCTTATTAAGT       -1

 M   A   R   T   L   A   L   Q   A   P   S   S   L   S   A   S   S   R       18
ATG GCT CGT ACA TTA GCT TTG CAG GCA CCC TCG TCG TTG TCA GCG TCG TCG CGA      54

 Q   H   S   F   A   S   S   T   S   T   L   R   L   S   S   G   D   I       36
CAA CAT AGC TTC GCG TCA TCA ACA TCA ACG CTC AGA CTG AGC AGT GGT GAC ATC     108

 G   R   G   L   F   A   I   V   I   L   L   L   R   M   S   S   V   Y       54
GGT CGT GGT CTA TTC GCC ATA GTT ATA CTA CTA TTG CGT ATG TCC TCC GTT TAT     162

 G   V   I   D   R   L   V   V   Q   T   S   S   G   P   V   R   G   R       72
GGC GTT ATC GAC CGT TTG GTG GTG CAG ACA TCA AGC GGT CCG GTG CGT GGT CGC     216

 S   V   T   V   Q   G   R   E   V   H   V   Y   T   G   I   P   Y   A       90
TCC GTT ACC GTA CAG GGT CGC GAA GTG CAC GTG TAT ACG GGC ATA CCA TAT GCC     270

 K   P   P   L   D   D   L   R   F   R   K   P   V   P   A   E   P   W      108
AAG CCA CCG TTG GAT GAT TTA CGA TTT CGC AAA CCG GTG CCA GCG GAA CCA TGG     324
                                                                  • 71
 H   G   V   L   D   A   T   R   L   P   A   T   C   V   Q   E   R   Y      126
CAC GGT GTG CTG GAT GCA ACT CGA CTG CCG GCA ACT TGT GTG CAA GAA AGA TAT     378
     • 73                                   • 84
 E   Y   F   P   G   F   S   G   E   E   I   W   N   P   N   T   N   V      144
GAA TAT TTT CCT GGT TTC TCA GGC GAA GAG ATA TGG AAT CCA AAT ACA AAC GTT     432

 S   E   D   C   L   Y   I   N   V   W   A   P   A   K   A   R   L   R      162
TCA GAG GAT TGC TTG TAC ATT AAT GTT TGG GCA CCA GCG AAA GCG CGT TTA AGG     486

 H   G   R   G   A   N   G   G   E   H   S   N   K   A   D   T   D   H      180
CAT GGA CGC GGC GCT AAT GGC GGT GAG CAC TCC AAT AAA GCC GAC ACC GAT CAT     540

 L   I   H   N   G   N   P   Q   N   T   T   N   G   L   P   V   L   I      198
TTG ATA CAT AAC GGA AAT CCG CAA AAC ACC ACA AAC GGC TTA CCC GTG CTT ATT     594
                                                                  • 130
     • 116
 W   I   Y   G   G   G   F   M   T   G   T   A   T   L   D   I   Y   N      216
TGG ATT TAT GGT GGT GGC TTT ATG ACC GGC ACT GCC ACA TTG GAC ATT TAC AAT     648

 A   D   I   M   S   A   V   G   N   V   I   V   A   S   F   Q   Y   R      234
GCG GAC ATT ATG TCC GCT GTG GGT AAT GTA ATA GTG GCT TCA TTT CAA TAT CGT     702

 V   G   A   F   G   F   L   H   L   S   P   A   M   P   G   Y   E   E      252
GTT GGC GCA TTT GGC TTC CTA CAT TTA TCG CCC GCC ATG CCT GGC TAT GAG GAG     756

 E   A   P   G   N   V   G   L   W   D   Q   A   L   A   I   R   W   L      270
GAG GCG CCC GGT AAT GTT GGC TTG TGG GAT CAA GCA TTG GCC ATA CGT TGG TTG     810

 K   T   N   A   H   A   F   G   G   N   P   E   W   M   T   L   F   G      288
AAA ACG AAT GCA CAT GCC TTT GGC GGT AAT CCG GAG TGG ATG ACA CTG TTT GGT     864
       200
 E   S   A   G   S   S   S   V   N   A   Q   L   V   S   P   V   T   A      306
GAA TCG GCT GGT TCG AGT TCG GTG AAT GCG CAG TTG GTG TCG CCA GTG ACG GCG     918
                                                                  • 233
 G   L   V   K   R   G   M   M   Q   S   G   T   M   N   A   P   W   S      324
GGT CTG GTG AAG CGT GGT ATG ATG CAA TCG GGC ACA ATG AAT GCG CCA TGG AGT     972
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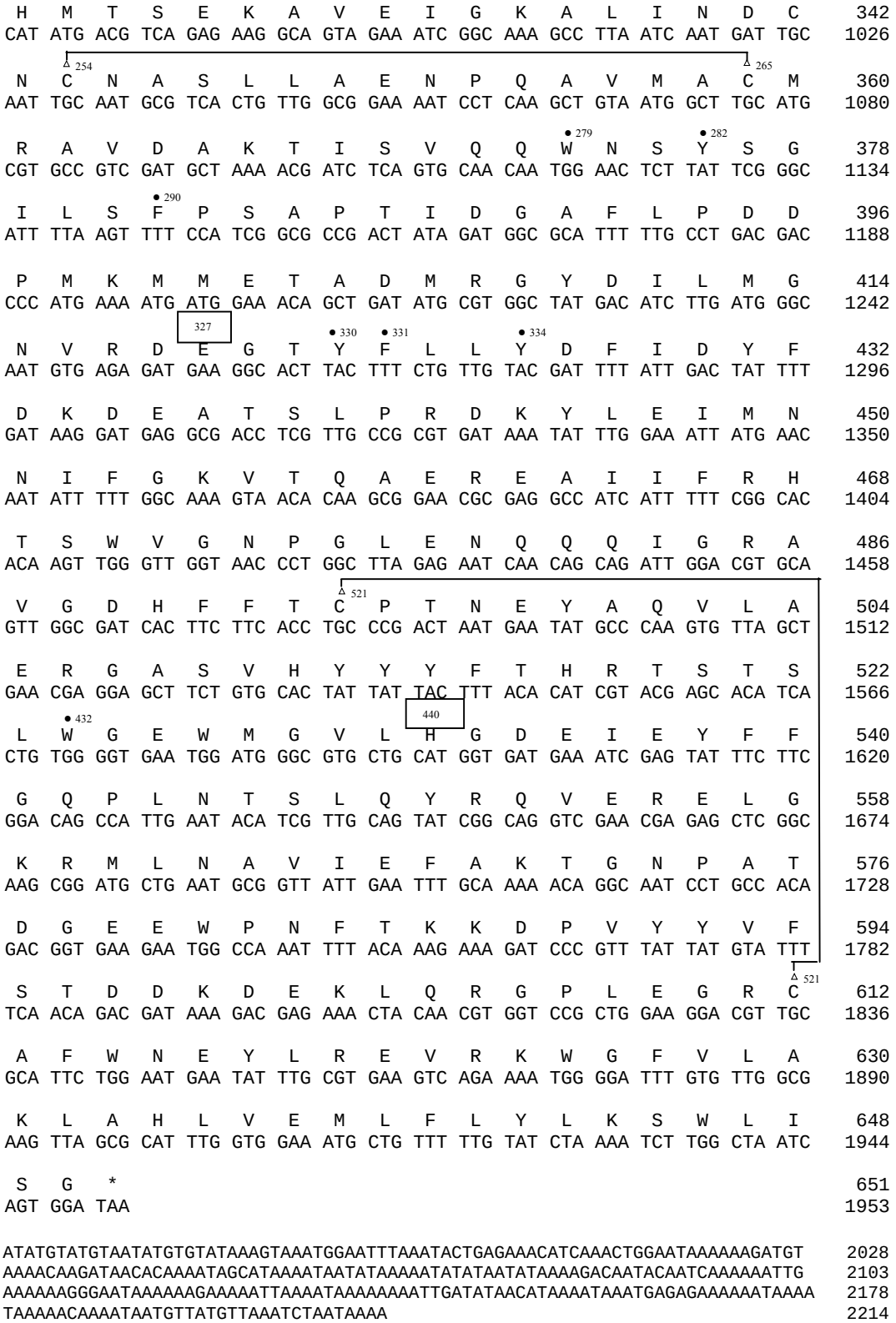

Figura 3 Alineamientos de la proteína AChE y la secuencia de nucleótidos de *C. capitata*. Parte superior la secuencia de aminoácidos y la secuencia de nucleótidos en la parte inferior. Los tres residuos que componen la tríada catalítica están indicados en números encuadrados (Ser200, Glu327 y His440) (el número del residuo es el que corresponde a la secuencia de *Torpedo*). Los

círculos rellenos indican 13 residuos aromáticos que intervienen en la afinidad del enzima por el sustrato y los círculos vacíos indican los residuos Gly118, Gly119 y Ala201 que ayudan a la estabilidad del complejo que se forma durante la catálisis. El penta-péptido Gly-Xaa-Ser-Xaa-Gly que está muy conservado se encuentra subrayado. Los triángulos representan las cisteínas involucradas en tres puentes disulfuro.

A nivel molecular, se investigó si la disminución en la susceptibilidad de la AChE a ser inhibida por malaoxon, en los individuos de fenotipo WR, era debida a cambios cuantitativos o cualitativos. Cuando se compararon las secuencias de cDNA de la AChE de tres individuos de cada uno de los tres fenotipos, se encontró una substitución en la base en la posición 1256 de una guanina por citosina, que produjo un cambio en el aminoácido encontrado en esa posición, una glicina por alanina. Esta mutación se encontró en homocigosis en los individuos WR, no estaba o se encontraba en heterocigosis en los individuos WS y no se encontró en ninguno de los individuos secuenciados pertenecientes a la línea C. Sin embargo no se encontraron diferencias en los niveles de expresión del mRNA de la AChE cuando se compararon, mediante PCR a tiempo real, los niveles de expresión del mRNA de la AChE de individuos de la línea W que habían sobrevivido a una concentración de 3000 ppm con individuos de la línea C.

Resistencia metabólica a malatión en *C. capitata*

El potencial de las enzimas metabólicas de ambas lineas, C y W, se ha determinado midiendo la actividad estarasa, citocromo P450 y glutatión transferasa. Especial atención se ha dado las esterasas y entre ellas a la ali-esterasa (αE7), que tiene un papel importante en la resistencia a malatión en moscas (Campbell *et al*., 1998a; Taskin y Kence, 2004; Taskin *et al.*, 2004).

Se han caracterizado los principales sistemas enzimáticos en adultos y larvas de la línea C de *C. capitata*. La tabla 7 muestra las actividades específicas de estos sistemas enzimáticos en diferentes tejidos de adultos (tubo digestivo medio, buche, cuerpo graso y tórax). Las mayores actividades específicas, se obtuvieron en el tubo medio de adultos. Independientemente del tejido utilizado, las actividades enzimáticas presentaron un máximo, a los pHs 7,5, 7, y 8 para las esterasas, P450 y GST, respectivamente.

Table 7. Caracterización de los sistemas enzimáticos de detoxificación en diferentes tejidos de adultos y el el tubo digetivo de la línea C de *C. capitata*.

Enzima[a]	pH	Actividad específica[b]				
		Larvas	Adultos			
		Tubo digestivo	Tubo digestivo		Cuerpo graso	Tórax
			medio	buche		
EST	7,5	82,4 ± 12	133,3 ± 8,4	87,7 ± 8,3	4,5 ± 0,4	9,9 ± 0,2
P450	7	8,9 ± 0,7	20,8 ± 0,2	7,6 ± 0,5	5,9 ± 0,04	1,3 ± 0,1
GST	8	78,0 ± 8,1	128,5 ± 5,0	67,4 ± 8,2	51,2 ± 1,6	29,3 ± 0,6

[a] EST, actividad esterasa usando α-naftil acetato (α-Na) como substrato; P450, citocromo P450 usando citocromo c como substrato; y GST, glutatión S-transferasa usando CDNB como substrato.
[b] Extracto del tubo digestivo de larvas y de diferentes tejidos de adultos fueron usados. Los valores son la media ± error estándar de tres réplicas experimentales. La actividad específica fue medida como nanomoles de α-Na hidrolizado/min/mg de proteína, nanomoles de citocromo c reducido /min/mg de proteína, nanomoles de CDNB conjugado/min/mg de proteína para esterasas, P450 y GST, respectivamente.

La actividad específica de las enzimas de detoxificación en ambas líneas, C y W fueron comparadas usando extractos de abdómenes individuales (Tabla 8). La actividad específica de P450, determinada por la reducción de citocromo c, y la actividad GST, determinada por la conjugación del CDNB (1-cloro-2,4 dinitrobenceno), no fueron diferentes entre las líneas C y W. Tampoco se encontraron diferencias significativas cuando se midió la actividad esterasa utilizando α-Na, β-Na, MUA o MUP como substratos. Sin embargo, la actividad esterasa determinada por la hidrólisis de MUH y MUB fue 2,5- veces mayor y 2,4-veces menor, respectivamente, en la línea W. Más importante fue la reducción de 1,4 veces en la hidrólisis del substrato MtB, encontrado en la línea W cuando fue comparada con la línea C.

Table 8. Actividad específica de las enzimas de detoxificación en las líneas susceptible (C) y resiste (W) de *C. capitata*.

Enzima[a]	Substrato	Actividad específica[b]	
		C	W
P450	Citocromo c	5,4 ± 0,5	4,2 ± 0,5
GST	CDNB	288,2 ± 13,9	288,9 ± 40,2
EST	α-Na	290, 2± 27,3	273,6 ± 29,2
	β-Na	298,9 ± 23,3	259,2 ± 34,2
	MUA	3,0 ± 0,2	3,2 ± 0,2
	MUB	2,9 ± 0,2	2,0 ± 0,2*
	MUH	3,2 ± 0,3	5,0 ± 0,4*
	MUP	0,07 ± 0,01	0,07 ± 0,01
	MtB	20,5 ± 2,0	12,4 ± 1,0*

[a] EST, actividad esterase usanda α-Na, β-naftil acetato (β-Na), metil umbeliferil acetato (MUA), metil umbeliferil butanoate (MUB), metil umbeliferil heptanoato (MUH), metal umbeliferil fosfato (MUP) y S-metil tiobutanoato (MtB) como substratos; P450, citocromo P450 usando citocromo c como substrato; y GST, glutatión S-transferasa usando CDNB como substrato.

[b] Los valores son la media ± error estándar de 10-25 individuos de cada línea. La actividad específica fue medida como nanomoles de α-Na, β-Na, MUA, MUB, MUH, MUP and MtB hidrolizado/min/mg de proteína, nanomoles de citocromo c reducido /min/mg de proteína, nanomoles de CDNB conjugado/min/mg de proteína para esterasas, P450 y GST, respectivamente.

[*] La media en la línea W fue significativamente diferente de la línea C ($p<0,05$, test de Mann-Whitney).

Se ha obtenido la secuencia codificante del gen ali-esterasa (*αE7*) a partir de individuos de la línea susceptible. Un primer fragmento se secuenció a partir de cebadores degenerados, diseñados en función de un fragmento muy conservado de la estructura primaria de la *αE7* de otras especies. A partir de este fragmento, se diseñaron cebadores específicos para la amplificación de los dos extremos 3' y 5' (Figura 4). La proteína muestras las principales características de las carboxil/colinesterasas.

```
GACATT                                                              -6

  M   Q   S   N   I   G   F   I   E   K   F   R   W   R   L   K   V   Y     18
ATG CAG TCA AAT ATT GGA TTT ATT GAA AAA TTC CGT TGG CGT TTA AAA GTC TAC     54

  E   H   K   Y   Q   Q   N   R   L   A   T   A   E   T   L   I   V   E     36
GAA CAC AAA TAC CAA CAA AAT CGA CTG GCC ACT GCA GAA ACG TTA ATT GTA GAG    108
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 T   E   Y   G   K   V   E   G   I   K   R   L   S   I   Y   N   I   P      54
ACT GAA TAT GGA AAA GTA GAA GGC ATT AAA CGC TTA AGT ATT TAC AAC ATT CCT    162

 Y   Y   S   F   E   G   I   P   Y   A   Q   P   P   V   G   E   L   R      72
TAC TAC AGC TTC GAG GGT ATA CCT TAT GCC CAA CCA CCT GTG GGT GAG CTA CGC    216

 F   R   A   P   Q   R   P   T   P   W   E   G   V   R   D   C   K   S      90
TTC AGA GCA CCT CAA AGG CCA ACT CCA TGG GAG GGT GTG CGA GAT TGC AAA AGC    270

 T   K   E   M   A   V   Q   T   H   I   I   T   G   I   L   E   G   S     108
ACC AAA GAA ATG GCG GTA CAA ACA CAT ATC ATA ACT GGA ATA CTG GAA GGA TCT    324

 E   D   C   L   Y   L   N   V   Y   T   N   N   T   L   P   D   K   P     126
GAA GAC TGT CTC TAC CTC AAT GTG TAT ACG AAT AAT ACT CTG CCT GAT AAG CCG    378

 R   P   V   M   I   W   I   H   G  **G** **G**  L   C   T  **G**  E   A   T     144
CGC CCA GTT ATG ATA TGG ATA CAT GGT GGT GGA CTT TGT ACT GGA GAG GCG ACA    432

 R   E   W   Y   G   P   D   Y   F   M   Q   K   D   I   V   L   V   T     162
CGT GAA TGG TAT GGA CCT GAT TAT TTC ATG CAA AAA GAT ATT GTG CTT GTG ACA    486

 M   Q   Y   R   L   G   V   L   G   F   L   S   L   G   T   P   E   L     180
ATG CAA TAT CGG CTA GGA GTA TTG GGC TTC CTT TCG CTG GGC ACA CCC GAA CTC    540

 N   V   P   G   N   S   G   L   K   D   Q   V   L   A   I   K   W   V     198
AAC GTA CCT GGA AAC TCT GGT CTG AAA GAC CAA GTA TTG GCT ATA AAA TGG GTG    594

 K   N   N   C   A   R   F   G   G   N   P   D   C   I   T   V   F   G     216
AAA AAT AAT TGT GCA AGA TTC GGT GGC AAC CCC GAC TGC ATA ACT GTA TTC GGT    648
     *
 E   S   A   G   A   T   S   A   H   C   M   M   L   T   E   Q   T   Q     234
GAA AGT GCT GGT GCG ACG TCT GCG CAT TGT ATG ATG CTC ACT GAA CAG ACA CAA    702

 G   L   F   H   R   A   I   L   M   S   G   T   A   L   P   L   W   E     252
GGT CTT TTC CAT CGC GCC ATT CTA ATG TCG GGT ACG GCG CTA CCC CTA TGG GAG    756

 T   E   D   Q   K   L   R   A   F   D   L   A   K   Y   A   G   Y   K     270
ACA GAG GAT CAA AAA TTA CGT GCT TTC GAT CTC GCA AAA TAC GCT GGA TAT AAG    810

 G   V   D   N   D   K   D   V   L   A   Y   L   R   K   C   K   A   K     288
GGT GTC GAT AAC GAT AAG GAT GTG TTG GCA TAT TTG CGC AAG TGC AAA GCG AAA    864

 D   L   I   A   L   E   G   R   T   L   T   A   E   D   R   A   R   N     306
GAT TTA ATT GCG CTC GAA GGT CGT ACA CTT ACT GCG GAG GAT CGT GCA CGT AAC    918

 I   S   T   P   F   V   Y   C   V   E   P   Y   V   T   P   E   C   V     324
ATA TCT ACG CCA TTC GTA TAT TGT GTA GAA CCG TAT GTG ACA CCT GAA TGT GTT    972

 I   Q   K   P   I   R   E   M   M   R   T   A   W   G   N   A   I   P     342
ATA CAA AAG CCG ATA AGG GAA ATG ATG AGA ACA GCG TGG GGT AAT GCG ATA CCG   1026
                             *
 L   L   V   G   H   A   S   D   E   G   L   I   F   L   Q   G   A   K     360
TTA TTA GTT GGT CAT GCG TCA GAT GAG GGG CTG ATC TTC TTG CAA GGC GCT AAG   1080

 I   L   A   S   I   A   Q   R   Q   K   S   Y   S   L   K   P   F   V     378
ATT TTA GCA AGC ATA GCC CAG AGA CAG AAA AGT TAT TCA TTA AAA CCA TTT GTA   1134

 P   Y   E   V   A   D   S   E   D   N   E   K   F   E   Q   K   L   R     396
CCT TAT GAA GTG GCG GAC AGC GAA GAT AAT GAA AAA TTT GAA CAG AAA CTG AGA   1188

 T   S   H   V   S   G   K   T   P   T   V   E   E   F   K   N   I   I     414
ACG TCG CAT GTG AGC GGC AAA ACT CCA ACA GTT GAG GAA TTC AAA AAT ATC ATC   1242
```

```
 A   Y   A   Y   L   H   F   P   L   Y   R   L   I   R   S   R   L   T     432
GCC TAT GCA TAT CTG CAC TTT CCA CTC TAC CGA CTA ATA CGC TCG CGC TTG ACA   1296

 Y   A   A   G   A   P   L   Y   L   Y   R   F   D   F   D   S   E   E     450
TAT GCG GCT GGC GCG CCC CTC TAC CTA TAT CGC TTC GAT TTT GAT TCC GAA GAA   1350

 L   P   H   P   Y   R   I   L   R   N   G   R   G   V   K   G   V   A     468
TTG CCA CAT CCC TAT CGT ATT CTA CGC AAT GGC CGT GGT GTA AAA GGC GTA GCG   1404
  *
 H   G   D   E   L   S   Y   I   F   T   N   L   F   S   C   T   L   S     486
CAT GGC GAT GAA CTT TCC TAT ATA TTT ACA AAC CTA TTT TCC TGC ACA TTA TCC   1458

 K   E   S   R   E   Y   R   T   I   E   R   M   V   G   F   W   T   Q     504
AAA GAG AGT CGT GAG TAT CGC ACC ATT GAA CGT ATG GTA GGC TTT TGG ACG CAA   1512

 F   A   Q   S   G   N   P   N   N   E   E   I   P   G   M   A   N   L     522
TTT GCA CAA AGT GGT AAT CCA AAT AAT GAG GAA ATT CCT GGT ATG GCA AAC TTA   1566

 T   W   D   P   L   K   K   S   S   P   K   L   N   C   L   N   I   S     540
ACA TGG GAT CCA CTG AAG AAA AGT TCA CCG AAA TTA AAT TGT CTG AAT ATA AGT   1620

 D   D   L   K   L   I   E   W   P   E   L   A   K   A   K   V   W   A     558
GAT GAT TTG AAA CTA ATC GAA TGG CCG GAA TTG GCC AAA GCG AAG GTG TGG GCA   1674

 N   A   Y   D   A   H   K   E   L   L   Y   *                         570
AAT GCT TAC GAT GCG CAT AAA GAA TTA TTG TAT TGA                         1710

AAAGGGGACAAAGGAGTGGTATAAAAAACACAAATATTGTCAAAAAACAATTCAATACAAAAGTAGTCATA
TCTGTGTAACTCAAAATGTGTATATATAGTATGTATTTTCTTAATTTATTTAAATACAATATATAATTTGT
TAATTATGCATTTGTTGTTTGTACATACTCGTATATTGTATTGGTTTATTAAAAAAAAATATTTTTTTACA
TATATATTTATATAGATTTGTGTATCAGGTAGTTAAATAGTAATCAATCTCATTAACCAAAATTCCACTTC
AAAAGCTTTATAAAACTATAAATTATGTACAAAATTTAAATAAATAAAACACAAAGGCTGCTTTCAACCAA
GCATGGAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Figure 4. Alineamientos de la proteína *αE7* y la secuencia de nucleótidos de *C. capitata*. Parte superior la secuencia de aminoácidos y la secuencia de nucleótidos en la parte inferior. Residuos que componen la tríada catalítica (*), residuos próximos a los que forman la tríada catalítica (subrayados), y residuos conservados en α-esterasas (PLYLYRFDFDS) en rojo.

La región condante del gen de la ali-esterasa (cDNA) se secuenció en individuos de ambas líneas C y W. En la comparación de ambas secuencias se encontraron 29 diferencias nucleotídicas. Sólo 6 diferencias resultaron en un cambio en el aminoácido en esas posiciones. Pero ninguno coincidió con los cambios estructurales que han sido relacionados con resistencia a OPs en *L. cuprina* y *M. domestica* (Campbell *et al*., 1998a; Claudianos *et al*., 1999). En cuanto al nivel de expresión de la *αE7*, no se encontraron diferencias significativas de expresión entre ambas líneas.

Discusión

En el presente estudio todas las poblaciones, recogidas en campos de diferentes zonas geográficas españolas y sometidas a una diferente presión de selección con malatión, mostraron una menor susceptibilidad a malatión (6 a 201-veces), cuando se compararon con dos poblaciones de laboratorio. Asimismo, las diferencias en susceptibilidad parecen estar correlacionadas con el número de tratamientos recibidos en cada una las zonas muestreadas. La poblaciones sometidas al mayor número de tratamientos (5-10 tratamientos/año), mostraron los mayores niveles de resistencia (LC_{50} entre 1000 y 3000 ppm y una LC_{50}>10000 ppm). Estos hechos son de gran relevancia para el control de esta plaga en la Comunidad Valenciana, ya que la concentración de malatión que se emplea en los tratamientos aéreos es de 7500 ppm. De hecho, fallos en el control pudieran estar produciéndose, ya que en los últimos años, el control de esta plaga ha requerido un incremento en el número de tratamientos.

Una línea resistente, W, procedente de la población recogida en Castellón, y una población susceptible de laboratorio, Lab-IVIA (C), fueron seleccionadas para posteriores estudios. Se analizó la toxicidad del malatión por ingestión y por vía tópica. En ambos casos, la línea W fue más resistente a malatión que la línea susceptible C, 22- y 79-veces, respectivamente. Estas diferencias entre los dos tipos de tratamientos podría deberse al modo en el que se suministra el insecticida en campo, en combinación con una proteína hidrolizada (malatión-cebo) que el insecto ingiere. Estos resultados contrastan con los obtenidos en los años 80 por Viñuela y Arroyo (1982), quienes no encontraron diferencias entre poblaciones de laboratorio y poblaciones españolas de campo en la susceptibilidad a malatión por vía tópica. Así, los resultados obtenidos por aplicación tópica con la línea C (DL50=1,2 µg/g) son semejantes a los obtenidos por Viñuela y Arroyo (1982) con las poblaciones de laboratorio (DL50=2 µg/g). Mientras que dosis (5 and 10 µg/g) que mataban el 100% de los individuos en las poblaciones de campo analizadas por Viñuela y Arroyo (1982) no causó la muerte de ninguno de los individuos de la línea resistente (W). Estos datos sugieren que la resistencia probablemente se haya desarrollado recientemente, por el uso continuado de malatión en campo para el control de *C. capitata*.

Los resultados de los ensayos de toxicidad, realizados con dos insecticidas permitidos para el control de *C. capitata,* mostraron que la línea W era aproximadamente 10

veces significativamente más tolerante a fentión que la línea C. Estos resultados podrían indicar que el mecanismo que confiere resistencia a malatión pudiera conferir resistencia a otros OPs (resistencia cruzada) o que, debido a que el fentión fue utilizado para el control de *C. capitata* durante los años 60s, 70s y 80s, dos mecanismos de resistencia se hubieran desarrollado independientemente (multiresistencia). En cuanto al spinosad, no se encontraron diferencias significativas entre ambas líneas (LC_{50}=0,4 ppm para la línea C y 0,7 ppm para W). Sin embargo, una línea de laboratorio de *Bactrocera dorsalis*, seleccionada para resistencia a malatión, mostró resistencia cruzada a spinosad (Hsu *et al.*, 2006), por lo que el potencial de desarrollo de resistencia a spinosad debe ser considerado si ambos insecticidas, malatión y spinosad, son usados conjuntamente en programas de manejo de *C. capitata*.

Se ha obtenido la secuencia condante del gen de la AChE (*Ccace*) a partir de individuos de la línea susceptible. Estudios filogenéticos muestran que *Ccace* es homólogo al gen de la AChE de otras especies de moscas secuenciados. Una mutación Gly328Ala (numeración de acuerdo con la numeración de *Torpedo*) en la AChE, parece estar asociada con la resistencia de *C. capitata* a malatión. Esta mutación está próxima a uno de los residuos de la triada catalítica en *D. melanogaster* (Walsh *et al.*, 2001), y se ha asociada con resistencia a insecticidas en poblaciones de *D. melanogaster*, *M. domestica* y *Plutela xylostela* (Walsh *et al.*, 2001; Menozii *et al.*, 2004; Baek *et al*., 2005; Lee *et al*., 2007). Nuestros resultados muestran que esta mutación se encontró en homocigosis en los adultos de la línea W con fenotipo WR y cuya AChE mostraba una menor sensibilidad a ser inhibida por el malaoxon, pero no al resto de los OPs ensayados. Estudios realizados en *D. melanogaster* y *M. domestica*, en los cuales se produjo la AChE que contenía esta mutación (Gly328Ala), mostraron que esta enzima mutada tenía menor susceptibilidad a ser inhibida por OPs pero especialmente por malaoxon (Walsh *et al.*, 2001; Menozii *et al.*, 2004). Los individuos con fenotipo WR, mostraron mayor k_m (menor afinidad) y una menor V_{max}, resultando en una reducción de 2,4 veces de la eficiencia (V_{max}/k_m) de la enzima. Similar reducción se ha mostrado en líneas resistentes de *M. domestica* y *P. xylostela* (Walsh *et al.*, 2001; Lee *et al*., 2007). Con respecto a los individuos de la línea resistente, que mostraban la mutación en heterocigosis (fenotipo WS), la AChE fue sensible al malaoxon y su actividad no estaba alterada. Esto puede indicar que la resistencia es recesiva. No obstante, la susceptibilidad a malatión de estos individuos debe ser analizada, ya que no siempre hay una correspondencia entre los ensayos *in vivo* y los

ensayos *in vitro*. Estudios que se están llevando a cabo en el laboratorio sugieren que esta resistencia es co-dominante, como ha sido indicado para otros casos de resistencia mediada por una modificación en la molécula diana (Bourguet *et al.*, 1996b).

El uso de sinergistas puso de manifiesto la posible implicación de las esterasas en la resistencia a malatión, ya que el DEF, un inhibidor de esterasas, incrementó la toxicidad del malatión 8,0-veces. Sin embargo, los resultados obtenidos tan solo muestran pequeñas diferencias en la actividad esterasa, entre la línea susceptible (C) y la resistente (W), cuando se utilizaron MUH, MUB y MtB como substratos. Estos resultados contrastan con las grandes diferencias encontradas en la actividad esterasa mostrada en especies donde una sobre-expresión de esterasas es la responsable de la resistencia a insecticidas, como en el caso de *Myzus persicae* (Field and Devonshire, 1998; Field *et al*., 1999; Blackman *et al.*, 1999) y *Culex pipiens* (Gullemaud *et al*, 1997). Las P450 (Maitra *et al.*, 2000) y GST (Taskin and Vence, 2004) también se han asociado con resistencia a malatión en moscas. Sin embargo, nuestros resultados de actividad enzimática y con sinergistas no indican que estos sistemas pudieran tener un papel importante en la resistencia. PBO, inhibidor de las P450, tuvo un efecto antagónico más que sinérgico del malatión, esto puede deberse a que al inhibirse las P450, no se produjo la activación de producto. Hemingway (1982) mostró que el PBO tubo un ligero efecto antagónico sobre el malatión en una línea resistente de *Anopheles stephensi*. Asimismo, el DEM, inhibidor de GST, disminuyó la toxicidad del malatión. Estudios realizados con este sinergista han mostrado que este inhibidor no es específico de GST y pude inhibir las P450 en algunas especies (Anders, 1978; Welling and De Vries, 1985).

Mutaciones puntuales en la E3 malatión carboxilesterasa de *L. cuprina* (Campbell et al., 1998a) y en el gen *αE7* de *M. domestica* (Taskin and Kence, 2004; Taskin *et al.*, 2004), que alteran la especificidad de la enzima, han sido relacionadas con la resistencia específica a malatión (MCE). En ambos casos estas mutaciones están relacionadas con un cambio en la especificidad del enzima, observándose una disminución en la actividad esterasa, medida con sustratos carbolxilester como el MtB, y un aumento en la capacidad de hidrolizar OPs. Los resultados obtenidos en este trabajo muestran una reducción significativa en la actividad esterasa usando MtB como substrato (1,6 veces) en la línea W, cuando se comparaba con la línea C. Sin embargo, la hidrólisis de malatión resultó muy baja en ambas líneas, y contrasta con lo que ocurre en otras especies donde este mecanismo de resistencia se ha

demostrado (Whyard et al., 1994; Campbell et al., 1998). Asimismo, el TPP, sinergista que se emplea para el diagnostico de este tipo de resistencia (Hughes et al., 1984; Hemingway, 1985), sólo incrementó ligeramente, 3,2- y 2,0-veces, la toxicidad del malatión en ambas líneas, W y C, respectivamente. Además, al comparar las secuencias codificantes del la αE7 (*Ccα E7*) en individuos de ambas líneas, no se encontraron ninguna de las dos mutaciones asociadas con resistencia a OPs en otras especies de moscas (Campbell *et al*., 1998a,b; Claudianos *et al.*, 1999; Taskin and Kence, 2004; Taskin *et al.*, 2004). No obstante, algunos individuos de la línea W presentaron cambios de aminoácidos en la secuencia de *Ccα E7*, que podrían estar relacionados con la disminución en la actividad esterasa. Por otra parte, no se encontraron incrementos significativos en el nivel de expresión del mRNA de la *α E7* en individuos de la línea W cuando se comparan con los de la línea C.

Conclusiones

1. Es la primera vez que se detecta resistencia al insecticida organofosforado malatión en poblaciones de campo de *C. capitata*. Las diferencias en susceptibilidad están relacionadas con el número de tratamientos recibidos, siendo las poblaciones que han estado sometidas a la mayor presión de selección con el insecticida, las de la Comunidad Valenciana, las que presentan los mayores niveles de resistencia.

2. Una línea resistente a malatión (W), procedente de la población de Castellón, mostró una menor susceptibilidad a fentión (9-fold) pero no a spinosad cuando se comparó con la línea susceptible de laboratorio (C).

3. Dos mecanismo parecen estar involucrados en la resistencia de *Ceratis capitata* a malatión, una modificación en la molécula diana y un mecanismo metabólico.

4. Las secuencias de la acetilcolinesterasa (AChE) y ali-esterasa han sido obtenidas por primera vez en *C. capitata*.

5. Una mutación Gly328Ala (numeración de acuerdo con la de *Torpedo*) en la acetilcolinesterasa (AChE) ha sido asociada con la resistencia de *C. capitata* a malatión. Adultos de la línea resistente que presentaron esta mutación en homocigosis fueron menos sensibles a la inhibición por malaoxon, sugiriendo

que una modificación en la AChE los permite sobrevivir a elevadas concentraciones del insecticida.

6. El sinergista trifenil fosfato (TPP) y S,S,S, tributil fosforotionato (DEF) incrementó la toxicidad de malatión por 3,2- y 8,0- veces, respectivamente, indicando que las esterasas podrían tener un papel importante en la resistencia.

7. Todos los individuos de la línea resistente mostraron una reducción en la actividad ali-esterasa, pero no se detecto hidrólisis in vitro del malatión ni se encontraron las mutaciones en la ali-esterasa que en otras especies de dipteros están relacionadas con resistencia a OPs. No obstante, se encontraron diferencias significativas en la actividad utilizando algunos substratos. Si estas diferencias están relacionadas o no con la resistencia de C. capitata a malatión debe ser determinado.

1 Introduction

The Mediterranean fruit fly (Medfly), *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is one of the most injurious fruit pests in the world. Different methods for the control of this pest are being implemented, but Pest Management plans are mainly focused on the application of chemical insecticides. Problems in controlling Medfly in the Comunidad Valenciana have resulted in an increase in the frequency of insecticidal treatments, especially the aerial application of malathion (Primo-Millo *et al.*, 2003). No cases of resistant Medfly populations have been reported up to date (Wood and Harris, 1989; Viñuela, 1998). However, the high selection pressure performed on some of these areas, as a consequence of the frequent treatments (5-10 times/year) with malathion-lure, may result in the development of resistance. This is particularly relevant, since the decision of non-inclusion of several insecticides in Annex 1 of the Directive 91/414/EEC, that regulates the process of revision of phytosanitary products in the European Community, may limit the number of available insecticides in the next years. Consequently, the risk of resistance development to malathion in Medfly and the possibility of cross-resistance to other insecticides must be investigated. Besides, the correct implementation of resistance management strategies, that prevent or delay the development of resistance, will require fundamental knowledge on the mechanisms of resistance. The understanding of these mechanisms would also provide the basis for the development of biochemical or molecular techniques for early detection of insecticide resistance that may allow oriented decisions in field treatments and the selection of the most useful insecticides.

1.1 The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann)

1.1.1 Taxonomical classification

Order: Diptera

Suborder: Brachycera

Infraorder: Cyclorrhapha

Family: Tephritidae

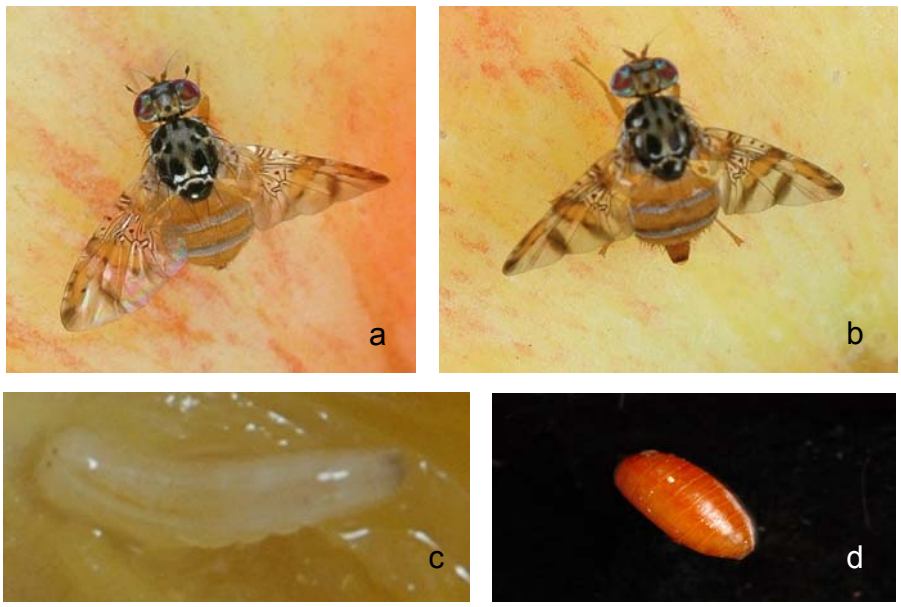
Genus: *Ceratitis*

Species: *Ceratitis capitata*

Ceratitis capitata (Wiedemann) (Diptera: Tephritidae). It was described by Wiedemann in 1824, as *Trypeta capitata*. The common name of the species is Mediterranean fruit fly or Medfly.

1.1.2 Description and geographical distribution

The adult Medfly is 4 to 5 mm long. The general color of the body is yellowish with a tinge of brown, especially the abdomen, legs and some of the markings on the wings. The scutellum is entirely black in apical half, with a sinuate yellow line across it sub-basally. The abdomen presents transverse yellowish and grey bands on basal half. The female can be distinguished by its long ovipositor at the apex of the abdomen. The larvae are elongate and yellowish white colored. Their anterior end narrows and is somewhat recurved ventrally, with anterior mouth hooks, and a flattened caudal end. Pupae are cylindrical, 4 mm long, dark reddish brown (Figure 1).



C. capitata: a) Adult male; b) adult female; c) larva; and d) pupa.

C. capitata has spread from its putative origin in East Africa to tropical and subtropical countries around the world (Fischer-Colibrie and Busch-Petersen, 1989; CABI, 1999). It was first reported in the Mediterranean area in the second half of the XIX century, and in the last century has spread to parts of Central and South America and Australia (Fischer-Colibrie and Busch-Petersen, 1989).

In Spain, Medfly was first seen near Malaga in the second half of the XIX century (Fischer-Colibrie and Busch-Petersen, 1989). Molecular studies suggest that the colonization of the Iberian Peninsula most probably occurred from south to north (Reyes and Ochando, 1998). At present, Medfly is widely distributed in Spain, including Baleares and Canary islands (Fimiani, 1989; Servicios de sanidad vegetal, 2004a,b), being especially abundant in the Mediterranean basin because of the favorable climatic conditions.

1.1.3 Biology

C. capitata is a multivoltine species, with short and overlapping generations (Bateman, 1972). In favorable areas, such as the south coast of Spain where the insect is present all year, the number of generations can be up to 7 or 8 (Ruiz-Castro, 1945; Muñiz and

Gil, 1984). The female lays the eggs under the skin of fruit that is just beginning to ripen. They usually deposit 1 to 10 eggs per clutch depending on the host, and may lay as many as 800 eggs during its lifetime (usually about 300) (Christenson and Foote, 1960; Papaj, 1990; McDonald and McInnis, 1985). When the eggs hatch, the larva promptly begins eating and tunnels into the pulp of the fruit. When the larvae reach the third instar, leave the fruit, drop to the ground and pupate in the soil. Duration of life cycle depends on temperature, which in optimal conditions lasts less than a month, but can be extended in cold temperature for 2-3 months (Christenson and Foote, 1960; Delrio, 1985). Wintering is normally passed as larval stage in fruits or as pupa in the ground. The seasonal pattern of abundance is determined by the climatic conditions and the availability of fruit hosts. In the temperate zone the population levels increase typically in the summer, reaching a peak in autumn and declining rapidly in the cooler season (Delrio, 1985).

1.1.4 Damage and economic importance

Medfly is considered one of the most destructive and widespread fruit pests in the world. Its host range includes more than 300 species of fruits and vegetables (Liquido *et al.*, 1991). Damage to fruit crops result from larval feeding that destroys the pulp, causing the premature drop of the fruits and providing the entry for bacteria and fungi. Indirect looses have also significant economic implications. Medfly is regulated as a quarantine pest in many countries. Thus, strict quarantine measures are imposed on fresh products from Medfly infested areas to prevent the entry of this pest. These measures range from extensive pre- and post-harvest monitoring and control procedures to a total ban on fruit import.

Medfly is an endemic pest in stone and citrus fruit crops of the Mediterranean area. The relevance of this species in our country is related to the importance of the citrus sector, being considered a pest of special agricultural incidence by the Agriculture Ministry since 1955 (Lloréns-Climent and Gilbert-Artiges, 1997). Spain is the main producer of citrus in the European Union with near to 6.3 million Tm of production in 271000 ha, and the first exporting country of fresh citrus world-wide (Anonymous, 2004). Direct losses in the production of citrus have increased in the last years, in spite of the permanent activities of control. Its incidence has been aggravated as a result of the expansion of extra early varieties of citrus, especially the Clementine "*Marisol*", extremely sensitive to the attack of this insect, because it reaches its total maturity at

the beginning of the fall, when temperatures are still high. In addition, the costs derived from the very restrictive protocols for the exportation of citrus fruits has aggravated the problem.

1.1.5 Control

The control of *C. capitata* mainly relies on the use of bait sprays. The development of eggs and larvae within the fruits protect them from non-systemic insecticides, being the adult the only life stage exposed to insecticides. Both males and females are strongly attracted to protein sources. In addition, females need a supply of proteins for sexual maturation and the production of eggs (Roessler, 1989). Thus, bait sprays containing an insecticide, usually malathion, and hydrolysed protein have been developed and successfully used for the control of Medfly adults (Roessler, 1989). However, the environmental and human health risks of chemical control have led, in the last years, to a search for alternative control methods more environmentally acceptable, such as mass trapping, the release of sterile males, and biological control. Cultural practices, such us removal of fallen and infested fruits and elimination of pupae by ploughing and digging, and control of isolated fruit trees (peaches, figs, pears, etc.) are also encouraged.

Control strategies in Spain involve field monitoring of the population levels and aerial and terrestrial treatments with organophosphorus insecticides (OPs). During the 60s, 70s and 80s the insecticide mostly used was fenthion but, it was replaced in the 90s by malathion when Spain began to export citrus fruits to United States (Lloréns-Climent and Gilabert-Artiges, 1997). Aerial and terrestrial treatments are performed using bait sprays, being necessary several insecticide applications (5-10) per year (Primo-Millo *et al.*, 2003). In addition, quarantine measures are also required for the exportation to Medfly-free areas.

Field monitoring

Considerable attention has been placed on detection methods of Medfly field populations to take treatment decisions in infested areas and to prevent their introduction to new areas. Different traps and attractants have been developed, being those based on male Medfly lures (sexual-attractant for males) or hydrolysed protein (food-attractant for both sexes) the most commonly used (Jang *et al.*, 2005; Heath *et al.*, 2004).

In the existing program in the Comunidad Valenciana, a total of 1131 traps were placed in 2006, covering most citrus areas. Monitoring is also required in areas of Cataluña, Murcia and Andalucía where citrus are produced for exportation to the United States. Monitoring is carried out with nadel type traps baited with a male lure (trimeldure) and a fumigant insecticide (vapona = dichlorvos).

Chemical control

Insecticides have been used to control *C. capitata* since the beginning of the 20th century (Roessler, 1989). First, inorganic insecticides were used (arsenate or sodium fluorsilicate), but they were replaced by chlorinated hydrocarbons (DDT, methoxychlor and dieldrin) after the Second World War. Thereafter, organophosphorous insecticides (OPs) came into use and continue being used today. Within the OPs compounds, malathion-bait sprays, which was introduced for the control of *C. capitata* in 1956, has been the most successful and widely used insecticide for the control of this pest throughout the world (Avidov *et al.*, 1963; Viñuela, 1998; Raga and Sato, 2005). More recently, pyrethroids, and biorational insecticides, such as spinosad and azadirachtin, are also available for the control of Medfly adults (Peck and McQuate, 2000; Burns *et al.*, 2001; Raga and Sato, 2005).

The insecticides registered for the control of *C. capitata* in citrus crops in Spain are listed in table 1.

Insecticides registered for controlling *C. capitata* in citrus crops in Spain.

Active ingredient	Formulated
Malathion[a]	25% [WP] P/P
	44% [EW] P/V
	90% [EC] P/V
	50% [EC] P/V
	118% (EQUIV. AL 97% P/P) [UL] P/V
Fosmet	20% [EC] P/V
	45% [SC] P/V
	50% [WP] P/P

Table 1. (Continued).

Active ingredient	Formulated
Trichlorforn	80% [SP] P/P
	50% [EC] P/V
	50% [SL] P/V
Fenthion	50% [EC] P/V
Spinosad[a]	0.024% [CB] P/V
Dichlorvos[b]	20% [EC] P/V
Lufenuron	3% [RB] P/P
Lambda cyhalotrin	10% [CS] P/V
	2.5% [WG] P/P
Azadiracthin	3.2% [EC] P/V

Font: Ministerio de Agricultura, Pesca y Alimentación. www.mapa.es

[a] Insecticide approved for exportation to United States

[b] Insecticide used only for mass trapping and monitoring

Malathion, fosmet, trichlorforn, fenthion and dichlorvos are organophosphorus compounds. Their main feature is an active phosphorous in the center of a phosphate [$O=P(OR)_3$], phosphonate [$O=P(OR)_2CR$], phosphorothionate [$S=P(OR)_3$], phosphorothiolate [$O=P(OR)_2SR$], phosphorothiolothionate [$S=P(OR)_2SR$], or related structures (Eto, 1974). They are essentially inhibitors of esterases including cholinesterases, being its target site the acetylcholinesterase (AChE) (Eto, 1974). Malathion is the most commonly used in both aerial and ground treatments. This insecticide was the first example of a wide spectrum organophosphorus insecticide combined with a very low mammalian toxicity. The molecule contains two carboxylesters bonds whose hydrolysis led to the detoxification of the insecticide. Its high selective toxicity is attributed to high carboxilesterase activity in mammals, in contrast with its low activity in susceptible insects (Eto, 1974).

Spinosad is a natural insecticide that consists in a mixture of spinosyns A and D, which are fermentation products of the soil actinomycete *Saccharopolyspora spinosa.* It is neurotoxic and acts as a contact and stomach poison (Salgado, 1998; Salgado *et al.*, 1998). The toxicity of spinosad against *C. capitata* has been demonstrated (Adan *et al.*, 1996; Stark *et al.*, 2004) and a formulation of spinosad (Spintor cebo, Dow Agrosiences Iberica S.A.) has been registered for its use as bait sprays in the last two citrus

campaigns. However, problems with the formulation were noted during field trials, and the product needs to be improved.

Lambda cyhalothrin is a synthetic pyrethroid. It disrupts the normal functioning of the nervous system in an organism by modifying the kinetics of voltage sensitive sodium channels which mediate the transient increase in the sodium permeability of the nerve membrane that underlies the nerve action potential (Soderlund *et al.,* 2002). Lambda cyhalothrin has been authorized for controlling *C. capitata* in the last two citrus campaigns, but its use is limited to a few areas.

Azadirachtin is a botanical insecticide derived from seeds of the neem tree, *Azadirachta indica*. It shows antifeedant effects (Butterworth and Morgan, 1971; Gil and Lewis, 1971) and insect growth regulator properties (Di Ilio *et al.*, 1999). Viñuela *et al.* (2000) and De Ilio *et al.* (1999) reported that azadirachtin was harmful to medfly because egg laying was almost completely inhibited. Di Ilio *et al.* (1999) showed the irreversibility of the process, while Viñuela *et al.* (2000) suggested that there was recuperation in egg laying when adults had ingested the insecticide only during the preoviposition period. Azadirachtin is authorized as toxicant in the traps for controlling *C. capitata* in biological agriculture.

Lufenuron is a phenylbenzoyl-urea that inhibits chitin synthesis and has a sterilizing action over male and females of *C. capitata* (Casaña-Giner *et al.*, 1999). It prevents eggs hatching from females that ingest the insecticide or females that mate with males that have ingested the insecticide.

Mass trapping

Trapping methods with special attractants may be used for controlling *C. capitata.* Traps baited with Tripack lures (ammonium acetate, putrescine and trimethylamine) (Heath *et al.*, 2004) and vapona as insecticide have proved to be effective in field trials (Katsoyannos *et al.*, 1999; Epsky *et al.*, 1999; Alonso and García Marí, 2004). This method is used, together with ground treatments, in places in the Comunidad Valenciana where aerial treatments are difficult due to the proximity of urban areas, roads or motorways, and for Medfly control in isolated fruit trees (Primo-Millo *et al.*, 2003).

The sterile insect technique (SIT)

The sterile insect technique (SIT) consists of mass rearing, sterilizing, and releasing sufficient numbers of competitive sterile insects to overflood the wild population over a significant geographic area (Gilmore, 1989). As only the males contribute to the induced sterility, genetic sexing strains [e.g., strains carrying a temperature sensitive lethal (tsl) mutation] are currently used in most sterile release programs, because of their increased effectiveness in suppressing pest populations (Hendrichs *et al.*, 1995, 2002). The insects are sterilized by gamma or X radiations that induce damage in the germinal tissue. Such a treatment induces dominant lethal mutations in the male sperm, which is then transferred to the female during mating (Hooper, 1989). The result is essentially a normal fertilization of the egg with functional sperm except that eggs fertilized with such sperm fail to hatch, thus the population starts to decline.

SIT programs against Medfly are currently used worldwide (Hendrichs *et al.*, 2002). In Spain, this technique was first used for the control of Medfly, in Tenerife Murcia and Granada during the 60s and the beginning of 70s (Mellado, 1971; Mellado *et al.*, 1974). A pilot program was initiated in 2003 in the Comunidad Valenciana for the release of sterile males imported from Argentina (Primo-Millo *et al.*, 2003). A sterile insect factory has been established in the Caudete de las Fuentes (Valencia) that is expected to produce 120000 millions of flies/week.

Strategies have also been developed for the attraction and sterilization of males and females of *C. capitata* by the chemosterilant action of the insect growth regulator, lufenuron, incorporated to a trapping lure (Casaña-Giner *et al.*, 1999). Field studies have shown that the chemosterilant traps significantly reduced the populations of *C. capitata* (Navarro-Llopis *et al.*, 2004, 2006), and they have been used in a pilot program that covered 3600 ha in Valencia during the last four years.

Biological control

Classical biological control primarily involves the importation and release of exotic parasitoids and predators (Wharton, 1989). Several parasitoids have been introduced for the biological control of *C. capitata* in Spain (García Marí *et al.*, 1994). During the 30s, two parasitoids, *Opius humilis* and *Diachasmamimorpha tryoni* were imported from United States. Then, this technique was replaced by the use of chemical control. Later, *Tetrastichus giffardianus* was introduced in the 60s and *Opius concolor* in the 70s. Albeit

none of these parasitoids showed an effective control of Medfly populations (García Marí *et al.,* 1994). Actually, two exotic parasitoids are being considered for their introduction in the Comunidad Valenciana: *Fopius arisanus* and *D. tryoni*. The egg parasitioid *F. arisanus* is native to South Asia (Wharton and Gilstrap, 1983), and has been introduced into many tropical regions, including Hawaii, Florida, Australia, Costa Rica, Fiji, and Italy (Snowball and Lukins, 1966; Purcell, 1998; Calvitti *et al.,* 2002). *D. tryoni* is native to Australia and attacks Medfly last instar larvae. It was introduced in Hawaii in the early 1910s, and shortly after was successfully established and contributed to the reduction of *C. capitata* populations (Wharton, 1989). Laboratory and semi-field trials have been already carried out in order to determinate its potential as control agents in Spain (Pérez-Hinarejos *et al.*, 2006; Santiago *et al.*, 2006), but their ability to adapt to the climatic conditions of the Mediterranean littoral are not determined yet

Local predators and parasitoids are important components of agro ecosystems that may also contribute to reduce the levels of Medfly populations. Ants, carabid and staphylinid beetles and spiders have been cited as preying on fruit fly larvae and pupae (Eskafi and Kolbe, 1990, Galli and Rampazo, 1996; Urbaneja *et al.*, 2006). However, few studies have evaluated their impact on tephritid populations (Debouzie, 1989). Recently, Urbaneja *et al.* (2006) have shown that predators are an important factor regulating Medfly populations in citrus orchards in Spain. Thus, appropriate management practices are recommended to maintain the levels of naturally occurring natural enemies.

1.1.6 Insecticide resistance

Resistance of *C. capitata* field populations to malathion has not been reported yet. (Wood and Harris, 1989; Viñuela 1998). Viñuela and Arroyo (1982) reported that Medfly populations collected from citrus and other fruit crops from different geographical areas in Spain were susceptible to malathion, trichlorform and diazinon. Moreover, in the last revision about the status of pesticide resistance in Spain, Viñuela, (1998) did not report control failures for this pest. It has been suggested that this may be due to incomplete selection pressure of the insecticide being used, caused by the mobility and broad-host range of *C. capitata* (Georghiou 1972; Orphanidis *et al.*, 1980). However, in the last years, the frequency of the treatments with malathion-lure has been gradually increased in some areas of the Comunidad Valenciana to maintain its effectiveness, which may be interpreted as the result of increased tolerance to

malathion. Albeit, the potential of *C. capitata* to develop resistance to malathion after intensive pressure by laboratory selection has been reported (Koren *et al.*, 1984).

1.2 Resistance to organophosphorous insecticides

Insecticide resistance occurs when an insect population becomes less sensitive to one or several insecticides, after a period of exposure to the compound(s). It involves heritable changes in a population, so that formerly useful pesticides no longer provide adequate control (Bernard and Philogene, 1993). At present there are more than 500 species of arthropods that have developed resistance to at least one type of insecticide (Hart and Pimentel, 2002). The experience on the evolution of resistance leads to the conclusion that, given an adequate pressure, nearly every species of insect is capable of developing resistance to a given insecticide. The essential requisites given rise to resistance are the presence of resistance genes and the selection pressure (Georghiou, 1972). Insecticide resistance results in economic losses, increased pesticide usage, and a greatly elevated potential for environmental contamination and human health problems.

Resistance to organophosphorous insecticides has been recognized in field populations of dipteran (de Carvalho *et al.*, 2006; Cui *et al.*, 2006), lepidopteran (Suckling *et al.*, 1984; Reuveny and Cohen, 2004), homopteran (Levitin and Cohen, 1998), and other insect (Conyers *et al.*, 1998) and mite (Herron *et al.*, 1998; Fitzgerald and Solomon, 2000) pests. The development of OP resistance has been studied in various Tephritid species around the world. Resistance to malathion has been reported for *Bactrocera dorsalis*, in Taiwan (Hsu and Feng, 2000), and for the fluit fly, *Dacus ciliatus*, in Israel (Maklakov *et al.*, 2001). Populations from Greece and Cyprus and a laboratory strain of *Bactrocera oleae* developed resistance to dimethoate (Vontas *et al.*, 2001, 2002a; Hawkes *et al.*, 2005; Skouras *et al.*, 2006). A laboratory strain from *B. dorsalis* developed resistance to fenitrohion after high selection pressure (Hsu *et al.*, 2006).

1 The resistance to malathion and other organophosphorous insecticides can be due to mutations on the target site, the acetylcholinesterase (Mutero *et al.*, 1994; Walsh *et al.*, 2001; Oakeshott *et al.*, 2005), or to the detoxification of the insecticides by metabolic mediated mechanism (Ranson *et al.*, 2002; Feyereisen,

2005). Acetylcholinesterase that is less sensitive to malathion may confer cross-resistance to other organophosphorous and carbamates (CB) insecticides, whereas metabolic resistance may result in even a wider range of cross-resistance by the inactivation of insecticides with different modes of action (Oakeshott *et al.*, 2005).

1.2.1 Target site resistance

1.2.1.1 Acetylcholinesterase

Acetylcholinesterase (acetyl choline acetyl hydrolase, AChE; EC 3.1.1.7) codified by *ace* gene, belongs to the very large group of enzymes called carboxyl/cholinesterases (Cygler *et al.*, 1993; Oakeshott *et al.*, 2005).

Two cholinesterases have been described in vertebrates, AChE and butyrylcholinesterase (BuChE, EC 3.1.1.8) (Eto, 1974; Oakeshott *et al.*, 2005). Both AChE and BuChE share extensive amino acid homology and display similar catalytic properties but differ in their function. AChE catalyzes the hydrolysis of the neurotransmitter acetylcholine, is subjected to marked substrate inhibition, and its main biological function is the termination of impulse transmission at cholinergic synapses of the central nervous system and neuromuscular junctions. BuChE prefers butiryl and propionylcholines as substrates rather than acetylcholine, shows no evidence of substrate inhibition, and it does not participate in nerve functions. AChE is found in nervous system, muscle tissue and erythrocytes, while BuChE is found in serum and in several organs, such as pancreas, hart and liver (Eto, 1974). Vertebrates have only one *ace* gene but, depending on the species, the *ace* gene produces different types of coding regions through the choice of 3' splice acceptor sites, defining distinct types of catalytic subunits generating proteins which posses the same catalytic domain associated with distinct C-terminal peptides: "readthroug" variant AChE(R); "hydrophobic" variant AChE(H); "tailed" variant AChE(T); and "soluble" variant AChE(S); differing in quaternary structure, solubility and the mode of anchoring to the surface membrane (Massoulié *et al.*, 1999; Massoulié, 2002). The AChE(T) is present in all vertebrates and represent the major type of acetylcholinesterase in cholinergic tissues. They produce a wide variety of oligomeric forms, ranging from monomers to heteromeric assemblies containing the anchoring proteins ColQ (collagen-tailed forms) and PRiMA (membrane-bound tetramers), which constitute the major functional enzyme species in mammalian muscles and brain respectively (Massoulié *et al*., 2005).

In insects, AChE is predominantly expressed as glycolipid (GPI)-anchored dimmers and mainly located in the central nervous system (Okeshott *et al.*, 2005). In contrast with vertebrates, neuromuscular transmission is not cholinergic in arthropods, being mainly based on glutamate and GABA as excitatory and inhibitory neurotransmitters (O'Brien, 1976; Fournier and Mutero, 1994). It is widely accepted that most insects and acari have two *ace* genes, termed *ace1* and *ace2* encoding AChE1 and AChE2, respectively, in the nomenclature of Weill *et al.,* (2003) [e.g. *Culex pipiens* (Bourguet *et al.*, 1996a; Malcolm *et al.*, 1998; Weill *et al.*, 2002); *Boophilus microplus* (Baxter and Barker, 2002); *Aphis gossypii* (Toda *et al.*, 2004); *Myzus persicae* (Nabeshima *et al.,* 2003); *Plutella xylostella* (Baek *et al.*, 2005); and *Blatella germanica* (Kim *et al.*, 2006)]. The only exception are higher Diptera that seem to have only one *ace* gene [e.g. *Drosophila melanogaster* (Fournier *et al.*, 1989; Mutero *et al.,* 1994); *Musca domestica* (Walsh *et al.,* 2001; Kozaki *et al.,* 2001); *Lucilia cuprina* (Chen *et al.,* 2001); *B. oleae* (Vontas *et al.,* 2002a); and *B. dorsalis* (Hsu *et al.*, 2006)] which shows high similarity to *ace2*. Only one AChE, AChE1, functions at cholinergic nerves, hydrolyzing acetylcholine in those insects that have two *ace* genes (Weill *et al.,* 2002). In higher Diptera, this function resides in their single AChE (Russell *et al.,* 2004).

The three-dimensional structure of AChE has been determined in vertebrates [*Torpedo californica Tc*AChE (Sussman *et al.,* 1991); mouse *m*AChE (Bourne *et al.*, 1995, 1999); and man *h*AChE (Kryger *et al.*, 1998)] and insects [*D. melanogaster Dm*AChE (Harel *et al.,* 2000)], allowing an understanding of reaction mechanism and the structural basis for mutagenic resistance to OP and carbamate insecticides. AChE belongs to the α/β hydrolase fold family, with a core of eight sheets connected by α-helices and stabilized by three disulfide bonds (Ollis *et al.*, 1992; Okeshott *et al.*, 2005). The main features of this family are: the catalytic triad (Ser200, Glu327 and His440, *T. californica* numbering), which is located near the bottom of a deep and narrow gorge, lined with aromatic residues (Sussman *et al.,* 1991); and a highly conserved Gly-Xaa-Ser-Xaa-Gly pentapéptide where the nucleophilic Ser of the calalytic triad is located (Oakeshott *et al.*, 1993).

The carboxyl/cholinesterase reaction proceeds in two steps. First, the oxygen of the serine residue of the catalytic triad makes a nucleophilic attack on the carbonyl carbon of the substrate (acetylcholine), displacing the alcohol (choline) and forming a relatively stable acyl-enzyme. The oxyanion formed in this transition state is stabilized through hydrogen bonding to the backbone nitrogens of three small residues that form the so-called oxyanion hole (Gly118, Gly119 and Ala201, *Torpedo* numbering). In the second

step water makes a similar nucleophilic attack, again forming a transition state intermediate, this time displacing the serine residue to release the acid product (acetic acid) and regenerate the free enzyme (Oakeshott *et al.*, 2005).

1.2.1.2 Inhibition of AChE by OPs

AChE is the target site for two of the largest groups of insecticides, organophosphate and carbamate compounds. These insecticides inhibit AChE disrupting its essential role of hydrolyzing the neurotransmitter, acetylcholine. The acetylcholine accumulates and its concentration remains at levels which are continuously too high preventing repolarization of the nerve cell, resulting in continuous firing of the nerve and the eventual death of the animal (Eto, 1974; Main, 1979). In the case of OPs, they form a phosphorylated enzyme intermediate instead of the acyl-enzyme intermediate that is formed with the choline ester substrate. The phosphoryl enzyme intermediate is far more stable than the acyl-enzyme and the regeneration of the enzyme is extremely low, presumably due to the inappropriate geometry of the phosphate group (O'Brien, 1976; Järv, 1984).

Many organophosphorus pesticides are generally poor inhibitors of esterases, unless they are converted into their active form. The activation is the transformation from the "thion" form (P=S, phosphorothionates and phosphorothiolates) to the "oxon" analog (P=O), which is the molecule active at the AChE site. This activation has been recognized to be performed by the cytochrome P450 monooxygenases (Eto, 1974). In the case of diazinon, this desulfuration has been studied for three heterologously expressed insect P450 enzymes (Dunkov *et al.*, 1997, Guzov *et al.*, 1998.; Sabourault *et al.*, 2001). However, it is not clear whether the activation of some OP compounds, such as disulfoton and fenthion, is catalyzed by P450 in insects (Feyereisen, 2005).

1.2.1.3 AChE mutations conferring insecticide resistance

Resistance to OPs can be due to alterations in the gene's regulation so that it produces more enzymes to overcome the effect of the insecticide or to structural changes in AChE that decrease the ability of the insecticide to physically bind to its site of action. Little evidence has been reported for the first one (Fournier *et al.*, 1992; Charpentier and Fournier, 2001), being the second, the main mechanism which confer resistance to OPs insecticides (Oakeshott *et al.*, 2005).

Russell *et al.* (2004) discerned two patterns of target site resistance to OPs and/or CBs. Pattern I is characterized at a bioassay level by much greater resistance to CBs than to OPs, and seen to be related to a change in the oxyanion hole. Pattern II resistance is characterized by more equivalent levels of resistance and/or reductions in AChE insensitivity to both classes of insecticides and it is related to the constriction of the active site gorge, limiting access of the pesticide to the catalytic residues (Mutero *et al.*, 1994; Levitin and Cohen, 1998; Walsh *et al.*, 2001; Rusell *et al.*, 2004). In table 2 are indicated those cases for which molecular data have been reported. A total 23 mutations have been found for 15 species of arthropods that involve 15 DmAChE-equivalent sites. Different patterns can originate from combination of various point mutations in AChE gene and high levels of AChE insensitivity could come from the combination together of several point mutations (Mutero *et al.*, 1994). All this mutations have been found in the cholinergic form of AChE (AChE2 for higher Diptera and AChE1 for the rest). The only exceptions are the mutations in AChE2 found in *Leptinotarsa decemlineata* (Zhu *et al.*, 1996) and *A. gossypii* (Li and Han, 2004), but their association with resistance is questioned nowadays.

A total of 14 mutations in AChE2 have been reported in four higher diptera *D. melanogaster*, *M. domestica*, *B. oleae* and *B. dorsalis* (Mutero *et al.*, 1994; Kozaki *et al.*, 2001; Vontas *et al.*, 2001, 2002a; Walsh *et al.*, 2001; Hsu *et al.*, 2006). They are located in just 10 *Dm*AChE-equivalent sites; mutations at 129, 227, 290, 328, and 396 (*Torpedo* numbering) occurring in more than one species. In tephritids, a total of three mutations have been reported Ile129Val, Gly396Ser and Gln591Arg. The Ile129Val substitution has been observed in a dimethoate resistant strain of *B. dorsalis* and in a fenitrothion resistant strain of *B. oleae* (Hsu *et al.*, 2006; Vontas *et al.*, 2002a) and is equivalent to the well characterized Ile129Val resistance associated mutation in *D. melanogaster* (Mutero *et al.*, 1994). In *B. oleae*, it was found in combination with the Gly396Ser substitution and in *B. dorsalis* in combination with Gly396Ser and Gln591Arg. The Gly396Ser substitution has been only reported in *B. dorsalis* and *B. oleae* (Hsu *et al.*, 2006; Vontas *et al.*, 2001, 2002a), this mutation may alter the configuration of the adjacent glutamate in the catalytic triad and promote the nucleophilic attack by water on the carbonyl group of the phosphorilated serine (Vontas *et al.*, 2002a). The third mutation, Gln591Arg, occurs near the end of the peptide and has been only observed in *B. dorsalis*. It was found in combination with the two others mutations Ile129Val and Gly396Ser (Hsu *et al.*, 2006).

Summary of mutations in AChE.

Position *Torpedo*[a]	Position *Drosophila*[b]	Comments[a]	Mutation[c]	Species	Comments[d]	References
73	73	At the entrance of the active site	Glu73(101)Gly	*Drosophila melanogaster*	Found in *Ace2*	Menozzi *et al.*, 2004
78[f,g]	78		Phe78(115)Ser[e]	*Drosophila melanogaster*	Found in *Ace2*. Pattern II of resistance	Mutero *et al.*, 1994
			Phe(139)Leu	*Aphis gossypii*	Found in *Ace2* (non-cholinergic AChE) Its implication in resistance is questioned	Li and Han, 2004
82	81	Near the entrance of the active site.	Glu81(119)Lys[e]	*Drosophila melanogaster*	Found in *Ace2*	Menozzi *et al.*, 2004
119[h]	151	Located in the oxyanion hole, alters the active site	Gly(247)Ser[e]	*Culex pipiens* *Anopheles gambiae* *Anopheles albimanus*	Found in *Ace1*. Pattern I of resistance	Weill *et al.*, 2003 Weill *et al.*, 2004
129[f]	161	Close to Trp84 which participe in the anionic site	Ile161(199)Val/The[e]	*Drosophila melanogaster*	Found in *Ace2*. Pattern II of resistance	Mutero *et al.*, 1994 Menozzi *et al.*, 2004
			Ile(214)Val	*Bactrocera oleae*	Found in *Ace2*. Pattern II of resistance Found in combination wiht Gly488Ser	Vontas *et al.*, 2002a
			Ile(214)Val	*Bactrocera dorsalis*	Found in *Ace2*	Hsu *et al.*, 2006
131	163	Located near the catalytic site	Asp(229)Gly[e]	*Plutela xylostela*	Found in *Ace1*	Lee *et al.*, 2007
150	182	Located in the active site gorge	Val180(260)Leu[e]	*Musca domestica*	Found in *Ace2*. Pattern II of resistance Not cause resistance by itself but, in combination with the double mutant Gly262Ala/Phe327Tyr or combine with Phe327Tyr enhance the insensitivity to insecticides	Kozaki *et al.*, 2001 Walsh *et al.*, 2001
201	239	Close to Ser200 (active site triad amino acid). Form the oxyanion hole in the active site of AChE	Ala(302)Ser	*Aphis gossypii*	Found in *Ace1* This mutation has been found in conjunction with the Ser331Phe mutation	Li and Han, 2004 Toda *et al.*, 2004 Andrews *et al.*, 2004
			Ale(298)Ser[e]	*Plutela xilostella*	Found in *Ace1*	Baek *et al.*, 2005 Lee *et al.*, 2007
227[f,g]	265	Affect the orientation of Ser200 (triad catalitic)	Gly265(303)Ala[e]	*Drosophila melanogaster*	Found in *Ace2*. Pattern II of resistance	Mutero *et al.*, 1994 Menozzi *et al.*, 2004
			Gly262(342)Ala/Val[e]	*Musca domestica*	Found in *Ace2*. They have been found in combination wiht Phe327Tyr	Walsh *et al.*, 2001 Kozaki *et al.*, 2001
			Gly205Ala	*Plutela xilostella*	Found in *Ace1*	Baek *et al.*, 2005

Table2 (Continued)

Position			Mutation[c]	Species	Comments[d]	References
Torpedo [a]	***Drosophila*** [b]	**Comments**[a]				
238	276	Locate far from the active site	Ser(291)Gly	*Leptinotarsa decemlineata*	Found in *Ace2* (non-cholinergic AChE) Its implication in resistance is questioned	Zhu *et al.*, 1996
290[f,g]	330	At the acyl pocket	Phe330(368)Tyr[e]	*Drosophila melanogaster*	Found in *Ace2*. Pattern II of resistance	Fournier *et al.*, 1992 Mutero *et al.*, 1994 Menozzi *et al*., 2004
			Phe327(387)Tyr[e]	*Musca domestica*	Found in *Ace2*. Pattern II of resistance	Walsh *et al.*, 2001
			Phe(416)Val[e]	*Culex pipiens*	Found in *Ace1*	Alout *et al.*, *2007*
			Phe(318)Val	*Cydia pomonella*	Found in *Ace1*. Pattern I of resistance	Cassanelli *et al.*, 2006
328	368	Near to the catalytic triad	Gly368(406)Ala[e]	*Drosophila melanogaster*	Found in *Ace2*. Pattern II of resistance	Menozzi *et al.*, 2004
			Gly365(445)Ala[e]	*Musca domestica*	Found in *Ace2*. Pattern II of resistance	Walsh *et al.*, 2001
			Gly(324)Ala[e]	*Plutela xilostella*	Found in *Ace1*	Baek *et al.*, 2005 Lee *et al.*, 2007
331	371	At the acyl pocket	Phe(445)Trp	*Culex tritaeniorhynchus*	Found in *Ace1*. Pattern II of resistance	Nabeshima *et al.*, 2004
			Phe(439)Cys	*Tetranychus urticae*	Found in *Ace1*	Anazawa *et al.*, 2003
			Phe(439)Trp	*Tetranychus kanzawai*	Found in *Ace1*	Aiki *et al.*, 2005
			Ser(431)Phe	*Aphis gossypii*	Found in *Ace1*	Toda *et al.*, 2004 Andrews *et al.*, 2004 Benting and Nauen, 2004
			Ser(431)Phe	*Myzus persiscae*	Found in *Ace1*. Pattern I of resistance	Nabeshima *et al.*, 2003
396	436	Close to Glu327 (active site triad amino acid)	Gly(488)Ser	*Bactrocera oleae*	Found in *Ace2*. Pattern II of resistance Mutation associated with the mutation Ile214Val	Vontas *et al.*, 2002a
			Gly(488)Ser	*Bactrocera dorsalis*	Found in *Ace2* .	Hsu *et al.*, 2006
	591	Near the end of the peptide	Gln(643)Arg	*Bactrocera dorsalis*	Found in *Ace2* . Occurs in combination wiht G488S and Ile214Val	Hsu *et al.*, 2006

[a] *Torpedo* numbering (Sussman *et. al.*, 1991).

[b] *Drosophila* numbering (Harel *et al.*, 2000).

[c] Number in brackets refers to the precursor protein.

[d] Pattern I: greater resistance to CBs than to OPs and pattern II: equivalent levels of resistance to OPs and CBs (Russell *et al.*, 2004). Residues referred to *Torpedo* numbering.

[e] Its involment in resistance has been verified by *in vitro* expression.

[f] Expresión of this mutation in *L. cuprina* AChE recombinant enzyme (Chen *et al.*, 2001).

[g] Expresión of this mutation in *Aedes aegypti* AChE recombinant enzyme (Vaughan *et al.*, 1997).

[h] Mutations at the same residue are known to alter substrate specificity in vertebrate butyrylcholinesterase (Lockridge *et al.*, 1997) and insect carboxylesterase E3 (Newcomb *et al.*, 1997) and in both cases OP hydrolysis is enhanced.

1.2.2 Metabolic resistance

Insects can metabolize toxic or otherwise detrimental chemicals, including insecticides, by complex multi-gene detoxification enzyme systems (Ranson *et al.*, 2002; ffrench-Constant *et al.*, 2004). The most common metabolic resistance mechanisms involve esterases, cytochrome P450 mooxygenases and glutathione S-transferses (Hemingway, 2000; Ranson *et al.*, 2002). Differences in activity of these enzymes are believed to be largely responsible for differences in insecticide toxicity between insect species (Croft and Mullin, 1984), between resistant and susceptible strains of the same species (Cariño *et al.*, 1994; Karunaratne and Hemingway, 2001; Huang and Han, 2007), and throughout the life stage (Suh *et al.*, 2000; Leonova and Slynko, 2004). Detoxification enzymes are induced in response to chemical stress (Terriere, 1984; Yu and Hsu, 1993) and can be detected in a wide range of tissues, but the highest activities are usually associated with the midgut, fat body and malpighian tubules, the most important sites for the detoxification of xenobiotics in insects (Scott, 1999; Enayati *et al.*, 2005).

Detoxification of OPs may be carried out by the hydrolysis at the phosphotriester bond that can be oxidative, mediated by cytochrome P450s or it can be hydrolyzed by the action of esterases (phosphotriester hydrolases), or it could even be mediated by glutathione transferases (Price, 1991). Additional sites of breakdown for malathion and a small number of other OPs (eg. phenthoate and acethion) are the terminal carboxylester groups, diethyl succinate in the case of malathion, that can be hydrolyzed by carboxylesterases (Price, 1991). This last mechanism has been demonstrated to be responsible for the low toxicity of malathion to mammals and for specific resistance to malathion in insects (Eto, 1974; Raftos, 1986). It has been attributed in some insects to enhanced degradation by a specific carboxylesterase, commonly referred to as malathion carboxylesterase (MCE), quite specific for malathion and related structures (Price, 1991; Raftos, 1986). Detoxification of OPs can also occur by esterases that are able to sequester these insecticides by binding permanently to the inhibitor molecule and preventing it from reaching its target site (Hemingway, 2000; Oakeshott *et al.*, 2005).

Synergists are natural or synthetic chemicals, considered non-toxic by themselves, which increase the lethality and effectiveness of insecticides (Bernard and Philogene, 1993). The mode of action of the majority of synergists is to block the metabolic detoxification systems that would otherwise break down insecticide molecules. For this reason, synergists are currently used in pesticide formulations to increase the efficacy of insecticides and overcome metabolic resistance (Bernard and Philogene, 1993). Synergists have also played a major role in the elucidation of resistant mechanisms in many arthropod species (Liu and Yue, 2000; Ahmad and Hollingworth, 2004; Espinosa *et al.*, 2005), since they specifically interfere with the different detoxification systems. The most commonly used are: the P450 inhibitor, piperonil butoxide (PBO) (Feyereisen, 2005); the Glutathione S-transferase inhibitor, dimethyl maleathe (DEM) (Mulder and Ouwerkerk-Mahadevan, 1997); and the esterase inhibitor, S,S,S-tributyl phosphorothioate (DEF) (Oakeshott *et al.*, 2005). Malathion resistance mediated by MCE has been often diagnosed on the basis of its synergism by triphernyl phosphate (TPP) (Oakeshott *et. al.*, 2005).

1.2.2.1 Esterases

Esterases are enzymes that can hydrolyze a diverse range of carboxylic-, thio-, phospho-, and other ester substrates. According to the International Union for Biochemestry and Molecular Biology (IUBMB) nomenclature, esterases are located within subgroup 1 of hydrolases (Enzyme Commission 3.1, EC 3.1), which is further categorized into subtypes based on the different types of ester bond hydrolyzed (www.chem.qmul.ac.uk/iubmb/enzyme/EC3/1/1/). Another common classification, that only consider esterase enzymes that preferentially hydrolyze carboxylesters, is based on their susceptibility to different inhibitors (the OPs, sulfhydral reagents, and the carbamate eserine sulfate), from which four classes of enzymes are discerned: i) acetylesterases, which are unaffected by any of these inhibitors and generally prefer substrates with acetyl acid groups and aromatic alcohol; ii) arylesterases, which are only inhibited by the sulfhydral reagents and generally prefer substrates with aromatic alcohol groups; iii) carboxylesterases, which are only inhibited by OPs and prefer aliphatic ester, generally of longer acids than acetate; and iv) cholinesterases, which are inhibited by OPs and eserine sulfate, and prefer substrates with charged alcohol moieties like choline esters over other aromatic or aliphatic esters (Holmes and Masters, 1967).

The esterases from different organisms have been cloned. Most of the eukaryotic esterases sequenced belongs to α/β hydrolase superfamily (Oakeshott *et al.*, 1993).

The main features of the primary sequence shared by esterases in this family are: a triad of non-contiguous residues, generally Ser-Asp-His, otherwise Ser-Glu-His or Cys-Asp-His; and the localization of the Ser/Cys nucleophile of the catalytic triad in a highly conserved Gly-Xaa-Ser/Cys-Xaa-Gly pentapeptide (Oakeshott *et al.*, 1993). Attending to the similarities between the sequences, esterases have been classified into six major families (Oakeshott *et al.*, 1993). One family contains several bacterial arylesterases, the dinelactone hydrolases. Then there are four major families of lipases and finally the largest and best characterized family called carboxyl/cholinesterase multigene family which includes some fungal lipases, some other eukaryotic carboxylesterases, all the cholinesterases, and all insect esterases so far sequenced (Okeshott *et al.*, 1993, 2005).

Two types of esterases have been implicated in resistance to OPs: phosphotriester hydrolases (phosphatases) and carboxylesterases.

Phosphotriester hydrolases: Phosphotriester hydrolases (EC 3.1.8) are enzymes with the ability to cleave the phosphotriester bond of OPs rather than being inhibited by them. This activity against OPs has been reported in houseflies (Welling *et al.*, 1971), lepidopterans (Konno *et al.*, 1989, 1990; Devorshak and Roe, 2001) and in *L. decemlineata* (Devorshak and Roe, 2001). Biochemical studies have showed the association of increase of phosphotriesterase activity with OP resistance in a parathion resistant strain of the tobacco budworm, *Heliotis virescens*, in an azinphos methyl resistant strain of the tufted apple bud moth, *Platynota idaeusalis* and in an azinphosmethyl and carbofuran resistant strain of *L. decemlineata* (Konno *et al.*, 1989; Devorshak and Roe, 2001). Phosphotriesterase has been purified from *P. idaeusalis* (Devorshak and Roe, 2001) and partially purified from *H. virescens* (Konno *et al.*, 1989), and they can hydrolyze paraoxon (Konno *et al.*, 1989; Devorshak and Roe, 2001). However, it is not known whether this mechanism involves increase in the amount of the enzyme or qualitative changes.

Carboxylesterases: Quantitative and/or qualitative changes in carboxylesterases (E.C. 3.1.1.1) may confer resistance to OPs by sequestering and/or hydrolyzing the insecticide (Wheelock *et al.*, 2005; Oakeshott *et al.*, 2005). The majority of carboxylesterases that function by sequestration are overexpressed through gene amplification, whereas those which produce resistance by hydrolyzing the insecticide are thought to occur by single point mutations in the structural genes.

High quantities of carboxylesterases, as a result of gene amplification, in resistant strains are able to sequester considerable amounts of OPs insecticides preventing the inhibition of the target site. This mechanism is responsible for the resistance against a broad spectrum of OPs in strains of the peach potato aphid, *M. persicae* (Field and Devonshire, 1998; Field *et al.*, 1999; Blackman *et al.*, 1999); the culicine mosquitoes (Mouches *et al.*, 1987; Gullemaud *et al.*, 1997; Hemingway and Karunamaratne, 1998; Qiao *et al.*, 1999); and in the brown planthopper *Nilaparvata lugens* (Small and Hemingway, 2000).

Alterations of the structure of carboxylesterases that confer to the enzyme ability to degrade OPs, but are associated with reduced aliesterase activity, was first described in 1960 by Oppenoorth and van Asperen, and is often termed the "mutant aliesterase" hypothesis. This mechanism appears to occur by single point mutations in the structural genes; although few have been characterized at molecular level. In two species of Diptera *M. domestica* (Claudianos *et al.*, 1999; Taskin and Kence, 2004; Taskin *et al.*, 2004) and *L. cuprina* (Campbell *et al.*, 1998a), this mechanism has been associated with two point mutations in the active site of the aliesterase *αE7* gene; Gly137Asp that coffer resistance to a broad spectrum of OPs and the mutation Trp251Ser/Leu that confer specific resistance to malathion (Campbell *et al.*, 1998a,b; Taskin and Kence, 2004). Newcomb *et al.* (1997) and Campbell *et al.* (1998a) proposed that the mutation Gly137Asp, which lies in the oxyanion hole, alters the orientation of this bound water molecule facilitating the dephosphorylation step of OPs hydrolysis, but compromising the ability to stabilize the tetrahedral intermediate formed with carboxyl substrates, explaining the loss of aliesterase activity. The resistance to malathion associated with the Trp251Ser/Leu mutation, in which a bulky aromatic residue is substituted by a smaller residue in the active site, appears to be due to a combination of MCE and OP hydrolase activities (Campbell *et al.*, 1998a; Oakeshott *et al.*, 2005).

1.2.2.2 Cytochrome P450 monooxygenases

Cytochromes P450 monooxygenases comprise a large family of enzymes that are found in all living organisms (Werck-Reichhart and Feyereisen, 2000; Feyereisen, 2005). They are best know for their monooxygenase role, catalyzing the transfer of one atom of molecular oxygen to a substrate and reducing the other to water, but they also show activity as oxidases, reductases, desaturases, isomerases, etc. P450 are named according to a nomenclature system where a CYP prefix, followed by an Arabic numeral that designates the family (>40% identity at the level of amino acid sequence), a capital letter that designates the subfamily (>55% identity) and an arabic numeral that designates the individual gene (all italics) or the protein (no italics).

A growing number of P450 (full length) sequences for insect species are available (http://P450.antibes.inra.fr). Ninety and 111 P450 genes have been identified in the genomes of *D. melanogaster* (85 genes and 5 pseudogenes) and *A. gambiae* (106 genes and 5 pseudogenes), respectively (Ranson *et al.*, 2002; Celniker *et. al.*, 2002; Holt *et al.*, 2002). They are distributed throughout 25 and 17 families respectively and more than half are members of CYP4 and CYP6 families (http://P450.antibes.inra.es). Danielson *et al.* (1999) sequenced 14 fragments of cytochrome P450s from *C. capitata*. Nine of the 14 fragments displayed the greatest positional identity to members of the CYP6A subfamily while the remaining five sequences exhibited maximum identity to CYP4A, CYP4D and CYP4E subfamilies.

Cytochromes P450 are involved in a large diversity of molecular and biological functions that include the metabolism of endogenous substrates and xenobiotic compounds. In insect, they play a great role in the metabolism of insecticides. In the case of organophosphorus insecticides, P450 are involved in both activation and degradation (Eto, 1974). The activation from the "thion" form to the "oxon" analog, which is the molecule active at the AChE site, is a P450-dependent reaction. In addition, the hydrolysis of the phosphotriester bond characteristic of OP insecticides and the carboxylester can be oxidative, mediated by P450 (Price, 1991, Feyereisen, 2005). The heterologous expression of insect P450 enzymes (CYP6A1 and CYP6A12) from *M. domestica* and *D. melanogaster* showed that diazinon was metabolized by both desulfuration and oxidative ester cleavage (Dunkov *et. al.*, 1997; Guzov *et. al.*, 1998; Sabourault *et. al.*, 2001).

The toxicology of OP compounds in insects is thus related to the balance between the activation and detoxification reactions (Price, 1991, Feyereisen., 2005). Changes in this balance may confer resistance to insecticides. Increase in oxidative ester cleavage over desulfuration was reported in a fenitrothion resistant strain of housefly (Ugaki *et al.*, 1985). Lower desulfuration/oxidative ester claveage ratio due to a decrease in desulfuration activity yielded resistance to methyl parathion in *H. virescens* (Konno *et al.*, 1989). In the case of malathion and related OPs, the oxidative cleavage of the carboxylester bond can also detoxified the insecticide. Welling *et al.* (1974) and Morton and Holwerda (1985) reported that cleavage of the carboxylester bonds by monooxygenases conferred resistance to malaoxon in *M. domestica* and *D. melanogaster*, respectively.

Molecular studies on various insects have shown that there is a positive correlation between insecticide resistance and overexpression of one or more *CYP* genes (Feyereisen, 2005). The exact molecular genetic basis of this overexpression is not completely understood. Studies of resistance mediated by P450 suggest that increase of expression is achieved through mutations and insertion/deletion in *cis*-acting promoter sequences and/or trans-acting regulatory loci (Feyereisen, 2005). *Cyp6a2* and *Cyp6a8* were constitutively overexpressed in a resistant strain to malathion (Maitra *et al.*, 2000). A strain of *Drosophila simulans* that overexpressed *CYP6G1* showed resistance to malathion (Le Goff *et al.*, 2003).

1.2.2.3 Glutathione S-transferases

Glutathion S-transferases (GSTs; EC 2.5.1.18) constitute a family of enzymes that participate in the detoxification of endogenous and exogenous toxic compounds (Enayati *et al.*, 2005; Ransom and Hemingway, 2005). These enzymes catalyze the conjugation of reduced glutathione (GSH) at its thiol group to any of a wide range of electrophilic compounds. Conjugation increases the solubility of the resultant products, thus facilitating their excretion (Habig *et al.*, 1974).

GSTs can be distributed into two groups based on their cellular localization; microsomal and cytosolic (Enayati *et al.*, 2005). These two groups are very different in structure and in origin but catalyze similar reactions. Only cytosolic GSTs are implicated in the metabolism of insecticides. Insect cytosolic GSTs were first classified into two immunological distinct classes of GSTs, classes I and II. Class I GSTs are encoded by a multigene family in *Anopheles* mosquitoes, *D. melanogaster* and *M. domestica*, whereas all class II GSTs are encoded by a single gene (Enayati *et al.*,

2005; Ransom and Hemingway, 2005). Later with the expansion of insect sequence data, additional GSTs were identified that did not fit into the class I or II. A new guideline based on greek letters was proposed for the naming of insect GSTs along the lines of the mammalian classification system (Enayati *et al.*, 2005; Ransom and Hemingway, 2005). Six classes (40% amino acid sequence identity) of cytosolic GSTs have been identified in both *A. gambiae* and *D. melanogaster* (Ransom and Hemingway, 2005). Delta and Epsilon classes are the largest classes of insect GSTs and have not been found in other organisms (Enayati *et al.*, 2005; Ransom and Hemingway, 2005), and play an important role in the detoxification of insecticides (Vontas *et al.*, 2002b; Ortelli *et al.*, 2003). Sigma class has been found in a diverse range of species from nematode to mammals, and all insect class II belong to this family (Enayati *et al.*, 2005; Ransom and Hemingway, 2005). Theta class has been found in many different species. Insect posses various theta GST genes but their role is still unknown. Zeta class has been also found in different species and their sequence is highly conserved, indicating that this protein is suggested to play an essential housekeeping role (Ransom and Hemingway, 2005). The Omega class has been found in different organisms, including insects, but its physiological function is unclear. One suggestion is that may play an important role in protecting against oxidative stress (Ransom and Hemingway, 2005).

In insects, GSTs have been studied for their role in detoxifying foreign compounds, mainly insecticides (Clark *et al.*, 1986; Vontas *et al.*, 2002b). The elevated GST activity has been associated with resistance to all the major classes of insecticides, such as pyrethroids (Vontas *et al.*, 2002b), DDT (Ranson *et al.,* 2001), and OPs (Kostaropoulos *et al.*, 2001). The conjugation of glutathione to organophosphate insecticides results in their detoxification via two distinct pathways depending on the chemical structure: I) In O-dealkilation, glutathione is conjugated with the alkyl portion of the insecticide, e.g. the demethylation of tetrachlorvinphos in resistant houseflies (Oppenoorth *et al.,* 1979); and II) in O-dearylation, the glutathione reacts with the leaving group, e.g. the detoxification of parathion and methyl parathion in *P. xylostella* (Chiang and Sun, 1993). Recombinant GST enzymes from the *P. xylostella* (PxGST3) and *M. domestica* (MdGST-6A) have verified the role of these enzymes in the metabolism of organophosphate insecticides (Huang *et al.,* 1998; Wei *et al.,* 2001). In addition, a malathion resistant strain of *M. domestica* in Turkey showed high GST activity (Taskin and Kence, 2004).

1.3 Aims of Thesis

The frequency of the insecticide treatments with bait sprays (malathion-lure) against the Mediterranean fruit fly has been gradually increased in the last years to maintain its effectiveness and fulfill the strict control measures imposed for the exportation of citric fruits to uninfected areas. This high selection pressure may lead to the development of resistance, a major practical problem associated with chemical control of pests. Moreover, selection with an insecticide may also confer cross-resistance to other insecticides, causing a serious impact on the control of insect pests by reducing effectiveness of many available insecticides.

In this context it is desirable to know the actual situation of field populations of *C. capitata* and study the potential resistance mechanisms to OPs to help us to design strategies to prevent or minimize the spread and evolution of resistance.

Specifically, the objectives of this thesis are:

1. To evaluate the susceptibility to malathion of Spanish field populations of *C. capitata* that have been subjected to different selection pressure during the last years. Synergistic effects and cross-resistance to other insecticides will be also studied.

2. To establish the role of target site insensitivity as a potential resistance mechanism to malathion in *C. capitata*. This study will cover the biochemical properties, sensitivity to oxon OP insecticides and the cDNA sequence of AChE in a resistant and a susceptible strain of *C. capitata*.

3. To determine the biochemical and molecular basis for metabolic resistance to malathion in *C. capitata*. The role of ali-esterases will be assessed by determining its substrate specificities and sequencing the *αE7* gene in the susceptible and resistant strains.

2 Resistance to malathion in field populations of *C. capitata*

The Mediterranean fruit fly (Medfly), *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is one of the most destructive pests of fruits in the world, attacking more than 300 species of fruits and vegetables (Liquido *et. al.,* 1991). In Spain, this fly is considered one of the most economically damaging pests of citrus orchards. Direct looses result from the oviposition in fruits, larval activity and eventual infection by fungi. In addition, quarantine measures are required for exportation to fly-free areas.

The control of *C. capitata* in the Comunidad Valenciana, which is the main area of citrus production in Spain, relays on field monitoring of the population levels and aerial and ground treatments with malathion bait sprays (Primo-Millo *et al.*, 2003). Resistance to malathion in the field has been reported for a number of insects (Perez-Mendoza *et al.*, 2000; Diaz-Pantoja *et al.*, 2000; Haubruge *et al.*, 2002), including some dipteran pests (Hughes *et al.*, 1984; Hemingway, 1985; Raghavendra *et al.*, 1998; Hsu and Feng, 2000). However, no resistance to malathion has been reported yet for field populations of *C. capitata* (Wood and Harris, 1989; Viñuela, 1998), despite being the most widely used insecticide for the control of this pest. It has been suggested that this may be due to incomplete selection pressure of the insecticide being used, caused by the mobility and broad-host range of *C. capitata* (Georghiou, 1972; Orphanidis *et al.*, 1980). Nonetheless, Koren *et al.* (1984) reported the potential of Medfly in developing resistance to malathion after 18 generations of selection in the laboratory. Besides, the frequency of insecticide treatments, in the last years, has been increased in some areas of the Comunidad Valenciana to maintain the effectiveness, which may be interpreted as the result of increased tolerance to malathion.

The aim of this study is to know the susceptibility to malathion of Spanish field populations of *C. capitata* that have been subjected to different selection pressure

during the last years. Effects of synergists, acethylcholinesterase activity and cross-resistance to other insecticides were also studied in a resistant and a susceptible strain in order to determine the nature of the resistance.

2.1 Material and methods

2.1.1 Collection and rearing of insects

Field sampling: Medfly adults were obtained from larvae-containing infested fruits collected in fruit orchard in 2004 or 2005. Sampling was performed at seven localities in the Comunidad Valenciana (Burriana, Castellón, Villareal, Carlet, Alcudia, Serra and Orihuela), one in Cataluña (Bajo Ampurdán) and other in La Rioja (Albelda). They were chosen attending to representative hosts (citrus and stone fruits in the Comunidad Valenciana, and apples in Gerona and La Rioja) and the insecticide regimes applied in those areas (Table 1).

Table 1. Sampling sites of *C. capitata* and spraying regimes.

Area	Locality	Year	Host	Malathion treatments against *C. capitata* [a]
Comunidad Valenciana	Burriana (Castellón)	2004	Medlar	8-9 a.t./year in 2003 and 2004
	Castellón (Castellón)	2004	Citrus	8-9 a.t. and 2-4 g.t./year in 2003 and 2004
	Villareal (Castellón)	2005	Khaki	urban area, non treated in the last 2-3 years
	Carlet (Valencia)	2004	Peach	8 a.t. in 2002, 6 at in 2003 and 6 at in 2004[b]
	Alcudia (Valencia)	2004	Peach	8 a.t. in 2002, 6 a.t. in 2003 and 6 a.t. in 2004[b]
	Serra (Valencia)	2005	Citrus	no treated in 2001, 2002, 2003 and 2004, 1 g.t. in 2005
	Orihuela (Alicante)	2005	Citrus	9 a.t. and 4 g.t. in 2005
Cataluña	Bajo Ampurdán (Gerona)	2004	Aple	1 g.t. in 2004
La Rioja	Albelda	2004	Aple	non treated field but close to other fruit crops treated

[a] a.t., aerial treatment, g.t., ground treatment.
[b] 4-6 additional ground treatments with fenthion.

The infested fruits collected in the fields were placed in plastic trays and located inside of a ventilated 5 l container, both containing a layer or vermiculite or arlite. They were kept in an environmentally controlled rearing room, at a photoperiod of 16 hours light and 8 hours dark, and a temperature of 26±3ºC, until pupation. Emerging adults from field collected fruits (F_0) were used for testing its susceptibility to malathion.

Laboratory Populations: Two laboratory populations were analyzed, Lab-INIA and Lab-IVIA. Lab-INIA was initially established at Instituto Nacional de Investigaciones Agrarias (INIA) of Madrid (Spain) and has been maintained for about 40 years in laboratory conditions. Lab-IVIA was established at Instituto Valenciano de Investigaciones Agrarias (IVIA) from wild *C. capitata* collected from non treated fields from the Comunidad Valenciana in 2001.

According to the results of the susceptibility bioassays, the susceptible strain Lab-IVIA (C), and a resistant strain established from the field population collected in Castellón (W) were maintained in the laboratory for further studies.

Lab-IVIA (C): The C strain has been maintained in our laboratory for the last three years under artificial conditions according to Albajes and Santiango-Álvarez (1980) with modifications. The adults were kept in rearing cages containing about 2000 individuals and fed with adult diet (4:1:0.1 glass sucrose: hydrolysate yeast: water). Water was supplied through a sponge wick fed from a water reservoir. The cages were cubical in shape (20x20x20 cm) with gauze on one side, through which the flies laid eggs that were collected in a try with water located below the cage (Figure 1a). The cages were kept in an environmentally controlled rearing room at the same environmental conditions indicated above.

The collected eggs were seeded at density of 0.15 ml of eggs in a container with 250 g of rearing diet (Table 2) that is covered with aluminum paper to avoid desiccation and placed in a 2 l plastic container with vermiculite at the bottom to allow third-instar larvae to exit from the food medium and pupate (Figure 1c). Six days prior to the eclosion (aprox. 14 days after seeding the eggs), pupae were sieved from the vermiculite and placed in the adult rearing cage (described above).

Table 2. Composition of the Rearing diet for *C. capitata* larvae.

Component	Amount	Provider
Deionised water	600 ml	
Brewer's yeast	36.3 g	Santiveri
Wheat bran	250 g	Santiveri
Sucrose	70 g	Merk
Prophyl paraben Nipasol	2.8 g	Sigma
Methyl paraben Nipagin	2.8 g	Sigma
Benzoic acid	2.5 g	Fluka

Castellón strain (W): The W strain has been maintained in our laboratory for the last three years. Adults were reared as described for the C strain but apples were regularly introduced (one or two per day) to the adult rearing cages as substrate for oviposition (Figure 1b). Apples were taken from the cage every 1-2 days and held on trays as shown in Figure 1d.

Each generation, except the sixth, the W strain was treated with malathion to maintain the selection pressure. About 1000-2000 adults (3-5 day-old) were treated with a concentration of malathion between 1000 and 3000 ppm, during 24 to 48 hours to obtain about 50% of mortality.

Figure 1. Rearing cages for: a) adults from the C strain, with a container below the cage to collect the eggs that the females lay through the gauze, b) adults from the W strain, with apples as substrate for laying eggs, c) larvae from the C strain reared on artificial diet; and d) larvae from the W strain reared on apples.

2.1.2 Bioassays

The arena for the bioassays consisted of ventilated plastic dishes (89 mm diameter, 23 mm high). Ten to twenty pupae were confined per plastic dish, and the emerged adults were fed with water and rearing diet for 3-5 days before testing (Figure 2). The insecticide was tested as follow: mixed with the diet (feeding bioassay), applied directly on the insect (topical application) or in combination with synergists (bioassays with synergists). Three to six different concentrations of insecticide with a partial response (mortality higher than 0% and below 100%) were used and two to four replicates were performed for each concentration. Flies were kept in an environmentally controlled rearing room during the tests, as the environmental conditions indicated above. Mortality was recorded after 48 hours. Medflies were considered dead if they were not able to walk.

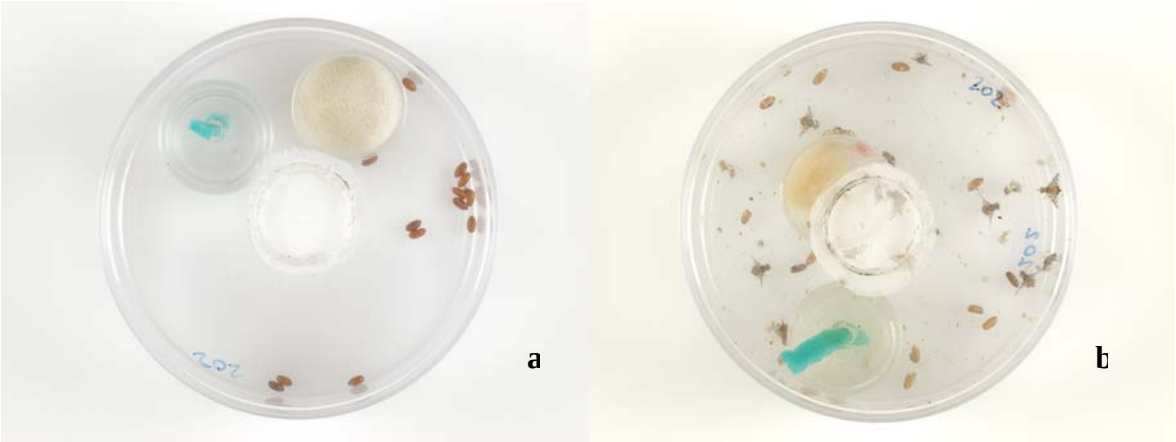


Figure 2. Ventilated plastic dishes used for the bioassays, containing water and adult diet in small plastic containers. Pupae (a) and emerged adults (b).

Feeding bioassays: Feeding bioassays were used to estimate the susceptiblility of field and laboratory populations to malathion and to examine the phenomenon of cross-resistance to fenthion and spinosad in the C and W strains. The insecticides used were: Malafin 50 EC (Agrodan S.A.), an emulsifiable concentrate of malathion 50% (w/v); Lebaycid LE (Bayer CropScience S.L.), an emulsifiable concentrate of fenthion 50% (w/v); and Spintor Cebo (Dow AgroSciences Ibérica S.A.), spinosad 0.024% (w/v). They were diluted in water and then mixed with the diet to obtain a range of concentrations. Three to five days after emergence of adults, the rearing diet was

replaced by diet containing the appropriate concentration of insecticide. The control consisted of diet mixed with water.

Topical application: Topical application was used to determine contact toxicity to malathion in the C and W strains. Malafin 50 EC was diluted in acetone to obtain a range of doses. Medflies were maintained at 4ºC for 30 minutes, thereafter a 0.5 µl drop of acetone solution of Malafin or acetone alone (control) was applied to the dorsal thorax of each Medfly (3-5 days old) by using an automatic microapplicator 900~x (Burkard). The treated insects were then placed in the ventilated plastic dish containing water and the rearing diet. Thirty randomly selected adults were weighed per bioassay to calculate the doses per fresh weight (µg insecticide/g fresh weight).

Synergists: Bioassays with synergists were used to elucidate the mechanism of resistance of the W strain to malathion. The synergists used were: DEF (S,S,S-tributyl phosphorotrithioate; 98% technical) as an esterase inhibitor, obtained from Chem. Service Inc.; TPP (triphenil phosphate, 98% technical) as inhibitor of ali-esterases, obtained from Fluka S.A.; PBO (piperonyl butoxide, 90% technical) as a cytochrome P450 (mooxygenases) inhibitor, obtained from Aldrich Chemical Co; and DEM (diethyl maleate, 97% technical) as inhibitor of glutathione S-transferase, obtained from Aldrich Chemical Co. Three µg of PBO, 1µg of DEM, 1µg of DEF or 5 µg of TPP was used per Medfly. These were the maximum doses of the synergists which could be used without any deleterious effects on the adult Medflies. Synergists were diluted in acetone and topically applied as described above. Acetone was used as control. After two hours, the flies were treated with malathion as described in the feeding bioassay.

Data analysis: Mortality data were used to determine their susceptibility to malathion (LC_{50} with a 95% confidence interval) by probit analysis using the computer programme POLO-PC (LeOra Software, 1997), which automatically corrected for control mortality by Abbott's transformation. The significance of differences in susceptibility was tested by the 95% confidence limits of lethal concentration ratios (LCR) at the LC_{50} (Robertson and Preisler, 1992).

The relative toxicity of malathion with respect to malathion plus the synergist (Potency ratio) was calculated using the computer programme POLO-PC.

2.1.3 Enzymatic assays

Tissue homogenate: Heads from twenty adults (3-5 days old) from the C and W strains were homogenized with a glass homogenizer in 600 µl of 0.15 M NaCl containing 1 % (v/v) of Triton X-100. Solubilized protein was isolated by centrifugation at 12000 rpm for 5 min in a UNIVERSAL 32 R (Hettich) centrifuge at 4ºC. The supernatant was collected and used as enzyme source.

Acetylcholynesterase (AchE) activity: AChE activity was determined by the spectrophotometric method described by Ellman *et al*. (1961). Three experimental replicates were carried out and blanks were used to account for spontaneous breakdown of the substrate. The reaction mixture consisted of 10 µl of tissue homogenate, 2 µl of 0.1 mM ASChI (acethylthiocholine iodiode, Sigma Chemical Co.), 2 µl 9.2 mM DTNB (5'5 dithio-bis (2-nitrobenzoic acid), Sigma Chemical Co.), and potassium phosphate buffer pH 7.2 up to 200 µl. The reaction was started by the addition of the substrate (ASChI) and the reagent (DTNB) and the increment in absorbance at 412 nm was recorded during 5 min. The AChE activity was converted to nanomoles of acetylthiocholine hydrolyzed per min and per mg of protein, using the extinction coefficient of the Ellman's product (ϵ_{412}= $1.36x10^4$ M^{-1} cm^{-1}) (Ellman, 1959).

Inhibition assays: Head extracts were incubated with $6.0\ 10^{-4}$, $9.0\ 10^{-4}$, $1.2\ 10^{-3}$ and $1.5\ 10^{-3}$ M of malathion (Malafin EC50) during 5 min at 30ºC. Three experimental replicates were carried out for each concentration and AChE activity was measured as describe above.

Protein concentration: It was determined according to the procedure of Bradford (1976) with bovine serum albumin (Sigma Chemical Co.) as protein standard. Protein standards were prepared in the proper buffer; maintaining the same concentration of Triton X-100 as in the samples (Friednauer and Berlet, 1989).

2.2 Results

2.2.1 Susceptibility to malathion in field and laboratory populations

Susceptibility of field and laboratory populations to malathion was tested by feeding bioassays in which the commercial formulated Malafin EC50 was added to the adult diet (Table 3).

Table 3. Susceptibility to malathion of field and laboratory populations of *C. capitata*.

Population	Field treatments with malathion	n	Slope ± S.E.	X^2	d.f.	LC_{50}[a] (95% CL)	LCR (LC_{50})[b] (95% CL)
Lab-IVIA	-	641	2.7 ± 0.3	88.6	33	16 (11 -19)	1
Lab-INIA	-	367	2.9 ± 0.3	29.8	18	12 (10-14)	0.8 (0.6 - 1.0)
Burriana	5-10 /year	185	0.8 ± 0.1	27.1	18	1376 (668-3126)	88 (47 - 166)*
Carlet	5-10 /year	148	1.7 ± 0.3	7.4	18	3137 (2182-4850)	201 (129 - 314)*
Orihuela	5-10 /year	136	0.8 ± 0.1	8.5	16	1636 (763-3629)	105 (46 -241)*
Alcudia	5-10 /year	95	1.2 ± 0.2	14.5	13	1029 (503-2074)	66 (34 - 127)*
Castellón	5-10 /year	208	0.9 ± 0.1	20.5	22	1874 (1190-3300)	120 (69 - 209)*
Bajo Ampurdán	1 /year	97	0.8 ± 0.2	23.0	23	519 (130-1169)	33 (11 - 100)*
Serra	1/year	95	0.9 ± 0.2	15.2	10	938 (278-2735)	60 (25 - 143)*
Villareal	non treated	251	1.6 ± 0.2	47.7	28	102 (44-160)	6 (4 - 11)*
Albelda	non treated	66	1.2 ± 0.3	10.5	10	472 (163-1084)	30 (13 - 72)*

[a] Concentrations expressed in ppm of malathion in the diet.

[b] Lethal concentration ratio (LCR) at LC_{50} level of each population with respect to the laboratory Lab-IVIA population.

*Lethal concentration is significantly different ($P<0.05$) if the LCR 95% confidence interval does not include 1.

All field populations were significantly less susceptible to the insecticide (LC_{50}>100 ppm of active ingredient) than the laboratory populations Lab-IVIA (LC_{50}=16 ppm) and Lab-INIA (LC_{50}=12 ppm), whereas no differences were detected between the two laboratory populations tested. More importantly differences in susceptibility were obtained between populations sampled in fields subjected to

different frequency of treatments. Those populations collected from fields with 5-10 aerial treatments/year showed the highest levels of resistance (LC_{50} between 1000-3000 ppm) with a lethal concentration ratio (LCR), with respect to the Lab-IVIA population, in the range 66-201. Populations from fields with only 1 treatment/year, Bajo Ampurdán and Serra, showed LC_{50} between 500-1000 ppm and LCR values between 33 and 60. Populations from untreated fields, Villarreal and Albelda, showed LC_{50} between 100-500 ppm and LCR values of 6 and 30, respectively. The mortality of the controls was always less than 5% (Table 3).

When the LCR values of all field populations were compared with respect to each other, the populations can be grouped according to the frequency of treatments (Table 4).

Table 4. Matrix of LCR at LC_{50} level to compare all field populations of *C. capitata* with respect to each other.

	Burriana	Carlet	Orihuela	Alcudia	Castellón	Bajo Ampurdán	Serra	Villareal	Albelda
Burriana	1								
Carlet	2.3 (1.2-4.4)*	1							
Orihuela	1.2 (0.5-2.9)	0.5 (0.2-1.2)	1						
Alcudia	0.7 (0.3-1.6)	0.3 (0.2-0.6)*	0.6 (0.2-1.6)	1					
Castellón	1.4 (0.6-2.8)	0.6 (0.3-1.1)	1.1 (0.5-2.8)	1.8 (0.8-3.8)	1				
Bajo Ampurdán	0.2 (0.1-0.5)*	0.16 (0.06-0.42)*	0.3 (0.1-0.9)*	0.5 (0.2-1.2)	0.2 (0.1-0.3)*	1			
Serra	0.7 (0.3-1.6)	0.3 (0.1-0.6)*	0.6 (0.2-1.6)	0.9 (0.4-2.2)	0.5 (0.2-1.2)	1.8 (0.6-5.4)	1		
Villareal	0.07 (0.04-0.14)*	0.03 (0.03-0.32)*	0.06 (0.03-0.14)*	0.09 (0.05-0.19)*	0.05 (0.03-0.1)*	0.2 (0.1-0.5)*	0.1 (0.05-0.24)*	1	
Albelda	0.3 (0.1-0.8)*	0.15 (0.07-0.32)*	0.3 (0.1-0.8)*	0.4 (0.2-1)	0.2 (0.1-0.6)*	0.9 (0.3-2.7)	0.5 (0.2-1.3)	4.6 (2.1-10.2)*	1

*Lethal concentration is significantly different ($P<0.05$) if the LCR 95% confidence interval does not include 1.

The populations with the highest number of treatments (Burriana, Carlet, Orihuela, Alcudia and Castellón) were not significantly different among them, except for the case of Carlet with Burriana and Alcudia. A second group includes the populations with only 1 treatment (Bajo Ampurdán and Serra) and the population from Albelda (collected from a non treated field but close to other treated fields). They were not significantly different from each other and presented LCR values lower than 1 with respect to the populations from the first group, although these differences were not always significant. Finally, the population from Villareal, obtained from an urban area that has not been treated in the last years, which was significantly different from all other populations.

2.2.2 Susceptibility of the resistant and the susceptible strains to malathion and other insecticides

The susceptibility to malathion of the resistant strain (W) derived from Castellón and the susceptible strain (C) from Lab-IVIA was assayed by ingestion and topical application (Table 5). Differences in susceptibility to malathion were obtained between both strains, independently of how the insecticide was applied. In both cases, the W strain was more resistant to malathion than the C strain, although the difference was higher by ingestion (LCR=79-fold) than by contact application (22-fold).

In addition, the susceptibility of the C and W strains to spinosad and fenthion were tested by ingestion (Table 5). Both spinosad and fenthion resulted more toxic for the C and W strains than malathion. However, the W strain (LC_{50}=12 ppm) was 9-fold significantly more tolerant to fenthion than the C strain (LC_{50}=1.3 ppm), whereas no significant differences were found with respect to spinosad (LC_{50}=0.4 ppm for the C strain and 0.7 ppm for the W strain).

Table 5. Susceptibility of the susceptible (C) and the resistant (W) strain of *C. capitata* to malathion, fenthion and spinosad.

Insecticide	Assay	Strain[a]	n	Slope ± S.E.	χ2	d.f.	LC_{50}[b] (95%CL)	LCR (LC_{50})[c] (95%CL)
Malathion								
	Feeding	C	162	2.3 ± 0.4	16.7	16	18 (11-23)	1
		W	210	0.9 ± 0.1	62.1	32	1406 (664-3150)	79 (44-141)*
	Topical	C	153	1.3 ± 0.2	24.4	15	1.2 (0.6-2.1)	1
		W	159	6.4 ± 1.4	39.5	17	27 (18-33)	22 (14-32)*
Fenthion								
	Feeding	C	108	1.8 ± 0.3	18.6	14	1.3 (0.6-2.2)	1
		W	144	2.6 ± 0.4	15.5	19	12 (10-15)	9 (6-16)*
Spinosad								
	Feeding	C	278	2.3 ± 0.1	13.7	10	0.4 (0.3-0.5)	1
		W	164	1.9 ± 0.4	27.1	18	0.7 (0.4-0.9)	2 (1-3)

[a] The susceptible Lab-IVIA (C) and the resistant Castellón (W) strains.

[b] Concentrations expressed in ppm of malathion, fenthion or spinosad in the diet for the feeding bioassays and as µg of malathion/ g fresh weight for topical assays.

[C] Lethal concentration ratio (LCR) at LC_{50} level of the resistant strain with respect to the susceptible strain.

[*] Lethal concentration is significantly different (P<0.05) if the LCR 95% confidence interval does not include 1.

2.2.3 Bioassays with synergists

Adults Medflies of the C and W strains were pretreated with synergists (TPP, DEF, PBO or DEM) two hours before being treated with malathion (Table 6) to block the main enzymatic systems involved in resistance. In the W strain, DEF (8.0-fold) and TPP (3.2-fold) significantly enhanced the effect of malathion, whereas no significant differences were obtained with DEM. On the other hand, there was an antagonism between malathion with PBO (0.3-fold). In the C strain, neither PBO nor DEF had any effect on the toxicity of malathion, DEM was antagonistic (0.6-fold) and TPP was moderately synergistic (2.0-fold).

Table 6. Effect of synergist on toxicity of malathion to the C and W strains of *C. capitata*.

Strain	Insecticide	Synergist	n	Slope ± S.E.	χ^2	g.l.	LC_{50}[a] (95% CL)	Potency[b] (95% CL)
C	malathion	-	319	2.4 ± 0.3	49.7	31	15 (11-19)	1
		+ TPP	148	2.1 ± 0.4	24.8	13	6.8 (2-11)	2 (1.3-3.4)*
		+ DEF	59	5.0 ± 1.6	1.0	4	16 (9-22)	1.14 (0.6-2)
		+ PBO	309	2.0 ± 0.3	96.6	30	18 (8-29)	0.8 (0.5-1.2)
		+ DEM	117	2.8 ± 0.5	7.1	10	25(19-33)	0.6 (0.4-0.9)*
W	malathion	-	568	0.9 ± 0.1	77.0	69	1303 (909-1881)	1
		+ TPP	133	0.9 ± 0.2	17.5	13	407 (177-1038)	3.2 (1.5-6.8)*
		+ DEF	108	0.8 ± 0.2	8.8	10	149 (25-407)	8 (3-21)*
		+ PBO	107	0.7 ± 0.2	20.6	12	5022 (1072 - 1037280)	0.3 (0.1-0.8)*
		+ DEM	49	0.8 ± 0.2	0.7	4	3491 (923 - 206410)	0.5 (0.1-1.6)

[a] Concentrations expressed in ppm of malathion.

[b] Potency of strain pretreated with synergist with respect to the same strain just treated with malathion.

*Potency is significantly different ($P<0.05$) if the confidence interval does not include 1.

2.2.4 AChE activity and inhibition by malathion

AChE activity and its inhibition by malathion was determined using pools of heads of *C. capitata* adults of the C and W strains. The specific activity (nanomoles of acetylthiocholine iodide hydrolyzed per min and per mg of protein) of the W strain (61.4±2.1) resulted lower than that of the C strain (80.2±2.1). In addition, the C

strain showed a higher decrease in enzyme activity than the W strain in the presence of increased concentrations of malathion (Figure 3).

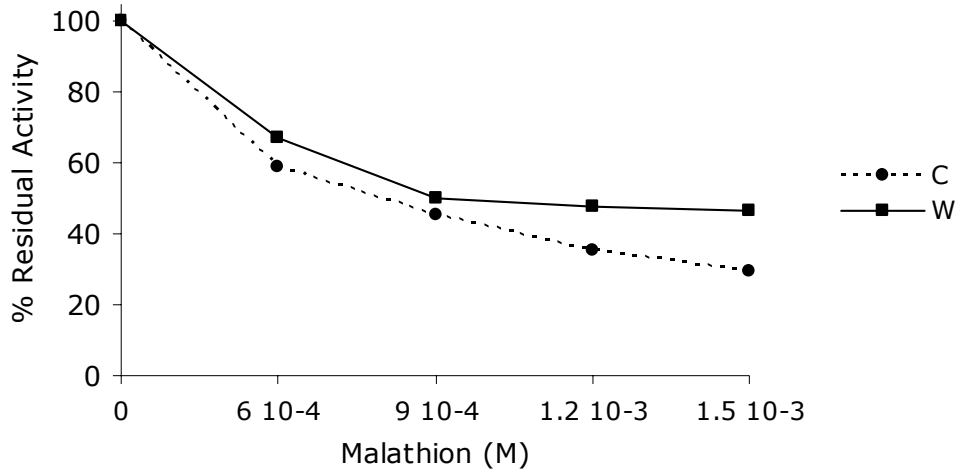


Figure 3. *In vitro* inhibition of AChE activity in C and W strains of *C. capitata*. The heads of 20 individuals from C and W strains were homogenized, pooled and assayed for residual acetylthiocholine iodide hydrolyzing activity under inhibition with different concentrations of malathion.

Discussion

In the present study, *C. capitata* field populations from citrus and other fruit crops from different geographical areas in Spain showed lower susceptibility to malathion (6 to 201-fold) when compared with laboratory populations. Moreover, differences in susceptibility can be correlated with the frequency of the field treatments. Accordingly, the five populations from the Comunidad Valenciana, subjected to the highest selection pressure (5-10 aerial treatments/year), showed the highest levels of resistance (LC_{50} between 1000-3000 ppm and LC_{90} over 10000 ppm). This finding is of great relevance for the control of this pest, in the Comunidad Valenciana, the main citrus growing area of Spain, since the concentration of malathion in the protein baits used in aerial treatments is 7500 ppm. In fact, control failures may be already occurring, which may have also contributed to the increase in the frequencies of the insecticide treatments experienced in the last years. Low levels of resistance (LC_{50} between 100-500 ppm) to malathion were also detected in field populations subjected to low selection pressure (1 ground treatment/year) or collected from fields that have

not been treated in the last years. Different studies indicate that *C. capitata* is a species in which the majority of adults do not move any great distances when hosts are available, but when hosts are absent or become unavailable in the area when the flies emerge, they will disperse rapidly and cover quite large distances in search of hosts (Fletcher, 1989). Thus, resistant flies may have dispersed from areas with high selection pressure to untreated areas.

A resistant strain (W), derived from a field population from Castellón, and a susceptible strain (C), from Lab-IVIA, were selected to compare the activity of malathion by ingestion and topical application. The W strain was more resistant to malathion than the C strain in both feeding (79-fold) and topical (22-fold) bioassays. The differences between both types of treatments may indicate that resistance evolved linked to the use of bait-sprays, since field exposure to the insecticide is mainly by ingestion. These results contrast with those obtained about twenty years ago by Viñuela and Arroyo (1982), who found no differences in susceptibility to malathion by topical application between laboratory and field populations of Medfly from citrus crops from the Comunidad Valenciana. Our results with the C strain by topical application (LC_{50} = 1.2 µg/g) are comparable to those obtained by Viñuela and Arroyo (1982) with laboratory populations (LC_{50} about 2 µg/g). However, Viñuela and Arroyo (1982) reported that doses of 5 and 10 µg/g resulted in 100% mortality in the field populations tested, whereas these doses produced no mortality in our resistant strain (data not showed). These finding suggest that, the detected resistance most probably developed recently because of the continuous use of malathion, as has been reported for the Oriental fruit fly, *Bactrocera dorsalis*, in Taiwan (Hsu and Feng, 2000).

Other insecticides approved for the control of *C. capitata* for citrus crops in Spain include fenthion and spinosad (http://www.mapa.es/es/agricultura/pags/fitos/fitos.asp), although their use is limited to a few areas. Our results showed that the W strain, compared with the C strain, was 9-fold more resistant to fenthion. It may indicate that the mechanism that confers resistance to malathion confers also moderate resistance to other OPs (cross-resistance) (Hsu *et al.*, 2004). Although, since fenthion was the most widely used insecticide for Medfly control in Spain during the 60s, 70s and 80s (Lloréns-Climent and Gilabert-Artiges, 1997), different mechanisms could have been selected independently to confer resistance to both insecticides (multi-resistance). With respect to spinosad, we have found no differences in susceptibility when the W and C strains were compared. Nevertheless, Hsu and Feng (2006) showed that a laboratory colony of *B. dorsalis* selected for resistance to

malathion or field populations collected from orchards in Taiwan, where malathion have been used for control purposes, showed cross-resistance to spinosad. Thus, the potential for resistance development must be considered if spinosad and malathion are used jointly in resistance management programmes. In any case, it is necessary to reduce the use of insecticides for the control of *C. capitata* and replace them for more environmental friendly strategies. In this sense, the Comunidad Valenciana has initiated pilot programmes to control *C. capitata* based on the release of sterile males and the use of insecticides with a chemosterilant action (Navarro-Llopis *et al.*, 2004).

The resistance to malathion and other OPs can be due to mutations on the target site, the acetylcholinesterase (Mutero *et al.*, 1994; Oakeshott *et al.*, 2005), or to the detoxification of the insecticide by metabolic enzymes (Ranson *et al.*, 2002; Feyereisen, 2005). We have found that the AChE activity of adult flies from the W resistant strain of *C. capitata* was less sensitive to inhibition by malathion, suggesting that target site insensitivity may be associated with malathion resistance. Modified AChE, showing a decreased in the sensitivity by OPs, has been linked with OP resistance in some insect species (Li and Han, 2004; Russell *et al.*, 2004) as well as in tephritids (Maklakov *et al.*, 2001; Vontas *et al.*, 2002a; Hsu *et al.*, 2006). Zahavi and Tahori (1970) reported that AChE in Medflies from a field population in Israel possessed an AChE less sensitivite to phosphamidon than flies from other field or laboratory strains, but they did not associate these differences with changes in the susceptibility to OPs insecticides. Moreover, we found a loss of AChE activity on the W strain when compared with the C strain, as has been reported for other cases of target-site resistance (Vontas *et al.*, 2002a; Hsu *et al.*, 2006). Using synergists, we have shown that the esterase inhibitor DEF partially suppressed the resistance to malathion (8-fold) in the resistant W strain, indicating that esterases may also play a moderate but significant role in the resistance to malathion in *C. capitata*. Increased detoxification of malathion by specific malathion carboxylesterase activity (MCE) is a well established resistant mechanism in many malathion resistant strains of different species (Townsend and Busvine, 1969; Hemingway, 1982; Hughes, *et al.*, 1984). This kind of resistance has been often diagnosed on the basis of its synergism by TPP (Hughes *et al.*, 1984; Hemingway, 1985; Baker *et al.*, 1998). Hughes *et al.*, (1984) indicated that TPP completely eliminated the resistance to malathion in a resistant strain of *Lucilia cuprina*, and the synergistic ratios exceeded 200-fold. Hemingway (1985) also indicated that a resistant strain of *Anopheles arabiensis* showed high levels of suppression of resistance by TPP. We have found that TPP slightly increased

the toxicity of malathion in both C (2-fold) and W (3.2-fold) strains. Thus, a hydrolytic mechanism mediated by esterases, but different to the MCE, seems to be involved in this resistance. However, inferences from the results with the synergists must be made cautiously since they are not entirely specific to a single detoxification enzyme class (Ahmad and Hollingworth, 2004). Additional biochemical and molecular studies are required to confirm this hypothesis.

Metabolic resistance to malathion mediated by cytochome P450s (Weeling *et al.*, 1974; Morton and Holwerda, 1985; Maitra *et al*., 2000) and glutathion S-transferases (Taskin and Kence, 2004) has also been described in dipterans. However, our results with synergists suggest that these enzymes are not involved in conferring resistance to malathion in *C. capitata*. In fact, the oxidase inhibitor PBO enhanced, rather than suppressed, the toxicity of malathion. This result may have been caused by the inhibition of the P450 mediated malathion-malaoxon conversion (0'Brien, 1976; Eto, 1974). Hemingway (1982) indicated that PBO had slightly antagonist effect on a malathion *Anopheles stephensi* resistant strain. The antagonistic effect observed was more pronounced in the resistant strain than in the susceptible one, in which PBO has only a slight effect. It may be due to two reasons: i) malathion, although is not the active form of the insecticide, is also toxic and it may have enough activity on the C strain, but not on the W strain which is about 100-fold more resistant; and ii) if esterases are involved in resistance in the W strain, an antagonistic effect would be expected when the activation of malathion is inhibited, since malaoxon is a potent inhibitor of esterases whereas malathion is a poor inhibitor of them (Oakeshott *et al.*, 2005). Interestingly, an antagonist effect on malathion toxicity was also obtained with DEM. It has been pointed out that DEM is not a completely specific inhibitor of glutation S-transferases and that it can inhibit cytochome P450s (Anders, 1978; Welling and De Vries, 1985). Thus, the antagonism of malathion by DEM could be also attributed to the inhibition of the metabolic activation of malathion.

In conclusion, resistance to malathion has been found, for the first time, in field populations of *C. capitata*. Both, target site insensitivity and metabolic resistance appear to be involved in the loss of susceptibility to this insecticide. Nevertheless, the resistance mechanisms need to be studied further to provide the necessary information for the design of a suitable resistance management strategy.

3 Target site resistance to malathion in *C. capitata*

One of the resistance mechanisms that confer resistance to insecticides in insects is the modification of the insecticide target protein (Morton, 1993; Fournier and Mutero, 1994). The target-site for organophophorous (OP) insecticides is Acetylcholinesterase (AChE, EC 3.1.1.7). This enzyme plays an essential role in neurotransmission at cholinergic synapses by catalyzing the hydrolysis of the neurotransmitter acetylcholine. OP insecticides covalently bind to the Ser of the active site and inhibit the enzyme (Aldridge, 1950). This inhibition leads to an accumulation of acetylcholine in the synapses preventing repolarisation of the nerve cell, resulting in continuous firing of the nerve, paralysis of the organism, and eventual death (Eto, 1974). After intensive use of OP insecticides in pest control, resistance mediated by alterations in the AChE has been developed in many insect species (Oakeshott *et al.*, 2005). Resistance to OPs due to alterations in the gene's regulation to produce more AChE to overcome the effect of the insecticide has been reported for *Drosophila melanogaster* and *Aonidiella aurantii* (Levitin and Cohen, 1998; Charpentier and Fournier, 2001). However point mutations in de AChE gene (*ace2* for higher Diptera and *ace1* for the rest of the insects) that make the enzyme less sensitive to inhibition by the insecticides, have more often been identified as being responsible for insecticide resistance (Mutero *et al.*, 1994; Zhu *et al.*, 1996; Huang *et al.*, 1997; Walsh *et al.*, 2001; Kozaki *et al.*, 2001; Vontas *et al.*, 2002a; Hsu *et al.*, 2006)

Intensive insecticide treatments with bait sprays (malathion-lure) against the Mediterranean fruit fly (Medfly) *Ceratitis capitata*, (Wiedemann) (Diptera: Tephritidae) has resulted in the development of resistant populations in some areas of the Comunidad Valenciana (Chapter 2). Our results indicate that populations from fields subjected to a high frequency of treatments were 2-30 fold more tolerant to malathion than populations from non-treated fields, and about 100 times more tolerant when compared to laboratory susceptible populations. In addition AChE activity of adult flies

from a resistant strain (W) of *C. capitata* was less sensitive to inhibition by malathion when compared with adult flies from a susceptible (C) strain, suggesting that target site insensitivity may be involved in the loss of susceptibility to this insecticide (Chapter 2). This study compares the biochemical properties and sensitivity of AChE to oxon OP insecticides in the W and C strains of *C. capitata*. The sequence of the AChE cDNA in both strains reveals the existence of one mutation in the resistant W strain.

3.1 Materials and methods

3.1.1 Insects

Two *C. capitata* strains have been maintained in the laboratory as described in section 2.1.1: the susceptible laboratory strain C and the resistant strain W derived from a field population collected in Castellón in 2004.

3.1.2 Enzymatic assays

Preparation of tissue homogenate: Heads from 3-5-days-old adults were homogenized in 0.15 M NaCl (one head in 600 µl), containing 1 % (v/v) of Triton X-100, with a glass homogenizer. Solubilized protein was isolated by centrifugation at 12000 rpm for 5 min in a UNIVERSAL 32 R (Hettich) centrifuge at 4ºC. The supernatant was collected and used as enzyme source. The protein concentration was determined according to the procedure of Bradford (1976) as described in section 2.1.3.

Acetylcholinesterase (AChE) activity: AChE activity was assayed using acetylthiocholine iodide (ATChI) as substrate, by the spectrophotometric Ellman´s method (Ellman *et al*., 1961), as described in section 2.1.3. The effect of pH in AChE activity was determined using pools of adult heads for individuals of the C strain. Reactions buffers were: 0.1 M potassium phosphate buffer (pH 6.0, 7.0, 8.0) and 0.1 M Tris-HCl (pH 8.0, 9.0, 9.5, 10.0).

AChE specific activity of single individuals for the susceptible (50 individuals) and the resistant (125 individuals) strains was also determined.

The kinetics parameters (k_m and V_{max}) of AChE were determined using pools of adult heads. AChE activity was assayed using six different concentrations of ATChI (10, 100, 250, 500, 750 and 1000 µM), and a double reciprocal plot was generated (Lineweaver-Burk plot). Three replicates were performed at each substrate concentration.

OP inhibitory assays: Malaoxon, chlorpiryfosoxon, azinphos-methyl-oxon and paraoxon were manufactured by Dr. Ehrenstorfer and provided by Cromlab S.L. These OPs are representative of the diethyl (paraoxon and chlorpiryfosoxon) and dimethyl (malaoxon and azinphos-methyl-oxon) classes of OPs. Stock solutions (0.1 M) of malaoxon, chlorpiryfosoxon and azinphos methyl oxon were prepared in ethanol and paraoxon was prepared in isopropanol and stored at 4ºC. Further dilutions were prepared in buffer immediately before use.

The kinetics of the reaction between AChE and the oxon OP inhibitors was investigated. Head extracts (pools) were incubated with different concentrations of inhibitor (malaoxon: 10^{-7}, $2.5x10^{-7}$, $5x10^{-7}$, $7.5x10^{-7}$, 10^{-6}, $2.5x10^{-6}$, $5x10^{-6}$ M; azinphos-methyl-oxon: $0.5x10^{-9}$, 10^{-9}, $0.5x10^{-8}$, 10^{-8} M; paraoxon: $2.5x10^{-7}$, $5x10^{-7}$, $7.5x10^{-7}$, 10^{-6} M; and chlorpiryfosoxon: 10^{-10}, $0.25x10^{-10}$, $0.5x10^{-10}$, $0.75x10^{-10}$, 10^{-9} M) at 30ºC. The concentration of the alcohol (ethanol or isopropanol) in the enzyme-inhibitor mixture was 1 % (v/v) or less; control samples contained no inhibitor but the same alcohol concentration. At suitable time-intervals (30 sec) the substrate (ASChI) and the reagent [5'5 dithio-bis (2-nitrobenzoic acid)] (DTNB) were added and the enzyme activity was measured as described in section 2.1.3. The experiment was performed three times. Plots were constructed, for each concentration of inhibitor, by plotting Ln of percentage of residual activity against time of incubation (0'Brien, 1976). The slopes, obtained by linear regression analysis, were first-order rate constant for each concentration of inhibitor. Reciprocal plots of inhibitor concentrations and the slopes calculated were constructed to yield the bimolecular rate constant (k_i) for each oxon OP (Main and Inverson, 1966).

3.1.3 Cloning and sequencing AChE cDNA from *C. capitata*

Total RNA was extracted from 100 adult heads (3-5 days old) of the C strain with TRIzol® reagent (Molecular Research Center). Heads were homogenized in a volume (V = 10x the weight of the heads) of TRIzol® reagent. Homogenates were incubated at

room temperature for 5 minutes. The mixture was centrifuged at 12000 g for 10 minutes at 4ºC. The aqueous phase was transferred to a new 1.5 ml microfuge tube and 1/5 V of chloroform was added and the mixture was mixed thoroughly by inverting the tube 4-5 times. The mixture was centrifuged at 12000 g for 15 minutes at 4ºC for phase separation. The aqueous phase was transferred to a new 1.5 ml microfuge tube, this step was done twice. The RNA was precipitated by adding 1/2 V of isopropanol and incubating 10 minutes at room temperature, and by centrifugation at 12000 g for 10 minutes at 4ºC. The pellet was washed with one V of 70% ethanol. The pellet was air dried for 10 minutes at 65ºC and resuspended in diethylpyrocarbonate (DEPC) treated deionised water. The total RNA was quantified spectrophotometrically according to Sambrook *et al.* (1989). Poly(A)$^+$ RNA was further purified from total RNA by using a mRNA purification Kit (Amershan Pharmacia Biotech. Ltd.), following the manufacturer instructions.

Single stranded cDNA from Poly(A)$^+$ RNA was synthesized, using the reverse transcriptase AMV (Roche). The 20 µl reaction contained 1 µg of Poly(A)$^+$ RNA, 1.7 µg of the primer AnchorT, 1mM of each dNTP, 0.8 units of AMV (Roche) in 1X manufacturer's reaction buffer and 0.4 µl of RNAsa inhibitor (invitrogen). The cycling conditions used were: initial desnaturation 65ºC for 5 min, 4ºC for 5 min, followed by 25ºC for 10 min, 42ºC for 60 min and 95ºC for 5 min. An internal cDNA fragment was amplified from the first strand cDNA using degenerated primers designed based on a region conserved in the AChE family (Table 1). A first PCR was conducted using Anchor and dFace2 (Table 1) as primers, and a second round of nested PCR was performed through a set of primers dFace_1 and dRace_1 (Table 1). PCRs were conducted in a DNA thermal cycler (Model 2400, Perkin Elmer). The 25 µl PCR reaction contained 2 µl of cDNA template, 0.4 µM of each primer, Anchor and Face_2 (Table 1), 3 mM $MgCl_2$, 0.2 mM of each dNTP and 4 units of AmpliTaq® DNA polymerase (Roche) in 1X manufacturer's reaction buffer. The PCR conditions were as follows: an initial desnaturation at 94ºC for 2 min; followed by 35 cycles consisting of 30 sec at 92ºC, one min at 54ºC, and 1.5 min at 72ºC; and a final step of 7 min at 72ºC to fully extend all PCR products. PCR products were separated by electrophoresis on 1-2% agarose gels and then gel-purified using Ultrafree-DA filters (Millipore) following the manufacturer's instructions. Purified amplicons were cloned into the pGEM-T Easy Vector System (Promega). DNA sequencing was performed at Secugen S.L on ABI PRISM 377 (Applied Biosystems, Perkin-Elmer).

After obtaining a cDNA fragment, sequence-specific primers (Table 1) were designed for rapid amplification of cDNA ends. The 5' end of the cDNA was amplified using Marathon cDNA as a template, whereas the 3' end was amplified by RT-PCR and direct sequencing of the PCR products.

Marathon cDNA was synthesized using Marathon cDNA amplification Kit (Clontech Laboratories Inc.) following the manufactured instructions. Briefly, 1 µg of head Poly(A)$^+$ RNA was used for the synthesis of the first cDNA strand, using a modified lock docking oligo (dT) with two degenerate nucleotide positions at the 3 end as a primer. Then, it was synthesized the double stranded cDNA and finally an adaptor was ligated to both ends. Two PCR were conducted to obtain the 5' end, for the first round of PCR, specific Race_2 and the Marathon adaptor were used as primers; and for the second PCR, the specific primer Race_2 and the nested primer were used (Table 1). In both cases, the 25 µl PCR reaction contained 0.1 µM of each primer, 0.2 mM of each dNTP and 0.5 µl of 50X BD^{TM} 2 Advantage Polymerase mix (BD Advantage^{TM} 2 PCR kit, Clontech) in the manufacturer's reaction buffer. The PCR conditions for the first round were as follows: an initial desnaturation at 94ºC for 2 min; followed by 35 cycles consisting of 30 sec at 92ºC, one min at 54ºC, and 1.5 min at 72ºC; and a final step of 7 min at 72ºC to fully extend all PCR products. The PCR conditions for the second round were as follows: 30 sec at 94ºC followed by 35 cycles consisting of 5 sec at 94ºC, two min at 68ºC. PCR products were purified, cloned and sequenced as described above.

The 3' end was obtained from a single adult individual from the C strain by RT-PCR. Total RNA was extracted from the head of a single individual with TRIzol® reagent (Molecular Research Center) as has been described above. 1µg of the total RNA was used as template for the first strand cDNA synthesis, that was performed with Reverse Transcriptase AMV (Roche) and AnchorT (Table 1) as a primer as has been described above. A first PCR was performed using Anchor and Face_4 as primers followed by a semi-nested PCR, using Anchor and Face3 as primers (Table 1). In both cases the 25 µl PCR reaction contained 2 µl of cDNA template, 0.4 µM of each primer, 3 mM $MgCl_2$, 0.2 mM of each dNTP and 4 units of AmpliTaq® DNA polymerase (Roche) in 1X manufacturer's reaction buffer. The PCR conditions for both PCRs were as follows: an initial desnaturation at 94ºC for 2 min; followed by 35 cycles consisting of 30 sec at 92ºC, one min at 54ºC, and 1.5 min at 72ºC; and a final step of 7 min at 72ºC to fully extend all PCR products.

DNA sequences were analysed using services provided by INFOBIOGEN (www. Infobiogen.fr; http://bioinfo.hku.hk/services/menuserv.html). The BLASTp algorithm was used to search the NCBI database for sequences similar to the *C. capitata* amplicons (Altschul *et al.*, 1990). Sequences were aligned using CLUSTAL v1.8.

Table 1. Primers designed to amplify AChE cDNA of *C. capitata*.

Primer[a]	Nucleotide sequence 5'-3'[b]
dFace_1	T[ATCG]TCTAGATGGAT[TCA]TA[TC]GGIGGIGG
dRace_1	[ATCG][CG]CTCGAGCIGCI[GC][AT][TC]TCICC[AG]AA
dFace_2	GGCATACCGTATGCCAAGCC
Face_3	ATGACCGGCACTGCCACATTGGACATTT
Race_2	CCACAAGCCAACATTACCGGGTGCCTCC
Face_4	CCTTTGCTGCGCTTTATTACAAC
Race_3	AAGGACGTTGCGCATTCTGGAATGA
Adaptor	CCATCCTAATACGACTCACTATAGGGC
Nested	ACTCACTATAGGGCTCGAGCGGC
AnchorT	GCCACGCGTCGACTAGTACGTTTTTTTTTTTTTTTTTTTTTT
Anchor	GCCACGCGTCGACTAGTACG
F1actinCc	CGGTTATTCGTTCACCACCA
R1actinCc	CTCAAAGTCCAAGGCGACA

[a] The suffixed d, F and R in primers names indicate degenerate, forward and reverse, respectively.

[b] I: inosine, used in degenerate primers.

3.1.4 RT-PCR and direct sequencing of AChE in single individuals

Total RNA was extracted from the thorax of individual flies from the C and W strains with TRIzol® reagent (Molecular Research Center) as has been describe above. In this case, total RNA was used as template for the first strand cDNA synthesis that was performed with Reverse Transcriptase AMV (Roche) and with AnchorT as a primer as has been explained above. A cDNA segment of 1904 bp was amplified by PCR; a first PCR was made using Anchor and the specific primer Face_4 and walked with a semi-nested PCR using the specific primers Face_4 and Race_3. For both reactions, the 25

µl PCR reactions contained 2 µl double-stranded cDNA, 0.4 µM of each primer, 3 mM $MgCl_2$, 0.2 mM of each dNTP and 4 units of AmpliTaq Gold® Taq (Roche) in the manufacturer's reaction buffer. The PCR conditions were as follows: an initial denaturation at 95ºC for 15 min; followed by 35 cycles consisting of 30 sec at 95ºC, 30 sec at 50ºC and 1 min at 72ºC; and a five min final extension at 72ºC. The PCR products were purified from the gel using QIAquick PCR Purification Kit (QIAGEN) and directly sequenced. Sequences were analyzed as has been described above.

3.1.5 Quantitative real time PCR (QPCR)

Total RNA was extracted from heads of single individuals of the C and W strains using TRIzol® reagent as described in section 3.1.3. First strand cDNA (20 µl) was synthesized from 1 µg of total RNA using Superscript kit (Invitrogen) following the manufactured instructions. The quantitative real time PCR amplification was performed using primers: Face_3 and Race_2 (Table 1) for the *ace* gene and F1actinCc and R1actinCc (Table 1) for the Actin gene of *C. capitata* (GenBank No. M76614) used as a reference gene. The amplification efficiency of each gene was estimated by using the equation $E=10^{-1/\text{slope}}$, where the slope was derived from the plot of amplification critical time (C_t value) versus serially diluted template cDNA. The optimized PCR master mix (15 µl) contained the following components: 5 µl of cDNA that has been diluted 10 fold with water as template, qPCR-TM Mastermix Plus for SYBR Green I (Eurogentec) and 3.6 µM of each gene specific primers. Optimized thermal program was: 1 cycle of 95ºC 10 min, followed by 40 cycles of 95ºC for 30 sec, 60ºC for 30 sec and 72ºC for 30 sec. QPCR reaction was performed using a continuous fluorescence detector, DNA Engine Opticon2 (Bio-Rad). Results were normalized to the actin mRNA level and calculated according to the ΔCt method. Each reaction was repeated three times to minimize intra-experiment variation. Analyses of the data were based on the average of all three replicates.

3.2 Results

3.2.1 Enzymatic activity, kinetics and inhibition of AChE from the susceptible and the resistant strains

Effect of pH on AChE activity: The activity was determined using ATChI as substrate, and presented maximum activity at pH ranging from 7.0 to 9.5 (Figure 1). Enzyme activity was poor under acidic conditions and also declined at pHs greater than 9.5. The enzymatic assays were measured at pH 7.2 since at higher pH the spontaneous degradation of the substrate was higher and interfered with the assay.

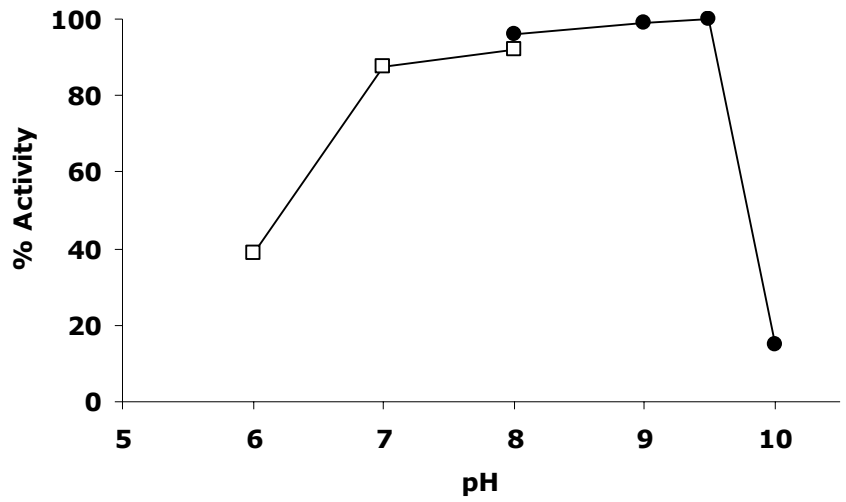


Figure 1. Effect of pH on AChE activity of adult head extracts of *C. capitata* using ATChI as substrate. □ 0.1 M potassium phosphate buffer ● 0.1 M Tris-HCl buffer.

AChE specific activity and susceptibility to malaoxon: The AChE specific activity of individuals from the susceptible C strain ranged between 60 and 190 nmoles min^{-1} mg^{-1} (Figure 2). However, the AChE specific activity of individuals from the resistant W strain presented a bimodal distribution with two distinct groups: one whose activity range was similar to the C strain (60 to 190 nmoles min^{-1} mg^{-1}) and a second group whose activity was lower (30 to 50 nmoles min^{-1} mg^{-1}) (Figure 2).

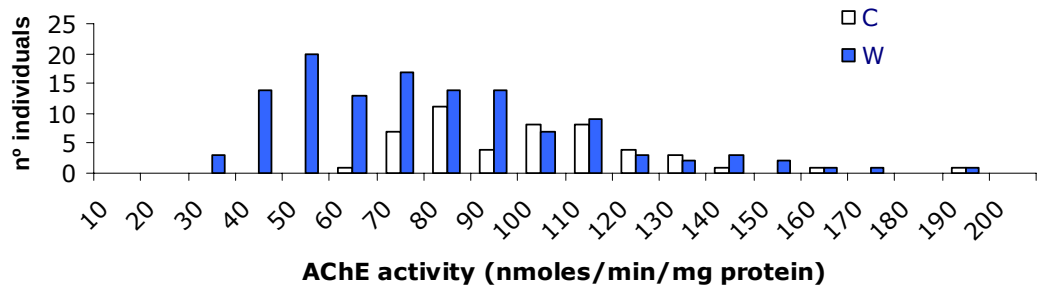


Figure 2. AChE specific activity from individuals of the susceptible C and the resistant W strains of *C. capitata*.

Sensitivity of AChE to inhibition by malaoxon was assessed in all these individuals. The head extracts were incubated for 5 min with a malaoxon (10^{-7} M) before starting the enzymatic reaction. This concentration produced about 50% inhibition of AChE activity in all individuals of the C strain and those individuals of the W strain with AChE activity over 60 nmoles min^{-1} mg^{-1}. However, AChE activity was not inhibited by 10^{-7} M in those individuals of the W strain with AChE activity lower than 60 nmoles min^{-1} mg^{-1}.

AChE Kinetic parameters k_m and V_{max}. Three pools of individuals, corresponding to the three AChE phenotypes identified, were made: i) individuals from the W strain with low altered AChE activity and no inhibition by malaoxon (WR); ii) individuals from the W strain with normal AChE activity and inhibition by malaoxon (WS); and iii) individuals from the C strain whose AChE activity was inhibited by malaoxon. AChE specific activity and kinetics constants for the three phenotypes are shown in table 2. AChE activity was higher in the phenotypes C and WS than in WR. In addition, the phenotype WR showed a reduction in V_{max} and higher k_m value, indicating that the altered AChE has affected its affinity for the substrate.

Table 2. Kinetic parameters of AChE from C, WS and WR phenotypes of *C. capitata*.

Phenotypes[a]	AChE activity[b] ± S.E. nmoles/ min/ mg	V_{max} ± S.E. nmoles/ min/ mg	K_m ± S.E. µM
C	82.2 ± 1.0	62.2 ± 1.2	77.8 ± 0.6
WS	91.2 ± 2.1	72.0 ± 0.5	77.9 ± 3.4
WR	45.4 ± 0.8	34.4 ± 0.0	105.8 ± 1.4

[a] Thirty individuals of each phenotype were pooled

[b] AChE activity using acetylthiocholine iodide as substrate. Mean of three experimental replicates ± standard error.

Sensitivity to OPs: Possible alterations of the AChE, resulting in a reduction in sensitivity to inhibition by OPs were investigated by determining the inhibition kinetics in C, WS and WR phenotypes. AChE activity was assayed with malaoxon, azynphos-methyl-oxon, paraoxon and chlorpiryfosoxon, which are the active forms of OP insecticides that irreversible inhibit AChE. With inhibitors in large excess, the pseudo first order kinetics was observed for the progressive inhibition of AChE. The apparent bimolecular rate constants of inhibition (k_i) was used to estimate the resistance levels of the *C. capitata* AChE (Table 3). The inhibition of AChE by malaoxon in the WR phenotype was lower than in the C and WS phenotypes, however no differences among the three phenotypes were found with the other oxon OPs.

Table 3. Inhibition of AChE from the C, WS and WR phenotypes of *C. capitata* by oxon OP inhibitors.

Phenotypes[a]	k_i[b] (10^7 $M^{-1}min^{-1}$ ± S.E.)			
	Malaoxon	Azinfos-metil-oxon	Paraoxon	Clorpirifosoxon
C	0.11 ± 0.02	6.7 ± 1.7	0.09 ± 0.01	76.6 ± 5.6
WS	0.12 ± 0.04	5.7 ± 2.2	0.09 ± 0.01	67.5 ± 4.8
WR	0.05 ± 0.01	5.7 ± 2.0	0.09 ± 0.01	58.3 ± 8.7

[a] Thirty individuals of each phenotype were pooled.

[b] Bimolecular rate constant of inhibition. AChE activity using acetylthiocholine iodide as substrate. Mean of three experimental replicates ± standard error.

3.2.2 Sequencing of the *ace* gene from *C. capitata*

A set of degenerate primers designed from conserved motifs of the AChE family produce an internal fragment of 300 bp. Cloning and sequencing, showed that this fragment exhibited similarity to known higher Diptera AChE2 proteins. The 5' end was amplified from Marathon cDNA using a Marathon and specific primers, and 3' end was amplify by RT-PCR. It resulted in a cDNA 2294 nucleotides long that contains an open reading frame that specifies a 669 amino acids polypeptide. The sequences from the clones, obtained from the Marathon cDNA, showed differences at eight positions, from which, 4 resulted in changes in the amino acid at those positions. It was found threonine or alanine, asparagine or aspartate, methionine or isoleucine, glutamate or glycine and isoleucine or valine at positions 190, 352, 413 and 436 in the predicted AChE protein from *C. capitata*, respectively. The full length sequence of the *ace* cDNA from *C. capitata* (*Ccace*) was also obtained from three individuals of the C strain by RT-PCR and direct sequencing using specific primers. In the comparison of the sequences, only three synonymous nucleotides differences have been observed, in one of the three individuals (Figure 3).

```
                                                                  AAATAT     -75
CCCAACCTACTCCTACCAACTAACCACCGTATCACCTACCACCCACTTCGACGAGCACACTCAATCTTATTAAGT      -1

 M   A   R   T   L   A   L   Q   A   P   S   S   L   S   A   S   S   R      18
ATG GCT CGT ACA TTA GCT TTG CAG GCA CCC TCG TCG TTG TCA GCG TCG TCG CGA      54

 Q   H   S   F   A   S   S   T   S   T   L   R   L   S   S   G   D   I      36
CAA CAT AGC TTC GCG TCA TCA ACA TCA ACG CTC AGR CTG AGC AGT GGT GAC ATC     108

 G   R   G   L   F   A   I   V   I   L   L   L   R   M   S   S   V   Y      54
GGT CGT GGT CTA TTC GCC ATA GTT ATM CTA CTA TTG CGT ATG TCC TCC GTT TAT     162

 G   V   I   D   R   L   V   V   Q   T   S   S   G   P   V   R   G   R      72
GGC GTT ATC GAC CGT TTG GTG GTG CAG ACA TCA AGC GGT CCG GTG CGT GGT CGC     216

 S   V   T   V   Q   G   R   E   V   H   V   Y   T   G   I   P   Y   A      90
TCC GTT ACC GTA CAG GGT CGC GAA GTG CAC GTG TAY ACG GGC ATA CCA TAT GCC     270

 K   P   P   L   D   D   L   R   F   R   K   P   V   P   A   E   P   W     108
AAG CCA CCG TTG GAT GAT TTA CGA TTT CGC AAA CCG GTG CCA GCG GAA CCA TGG     324
                                                  G67                 A71

 H   G   V   L   D   A   T   R   L   P   A   T   C   V   Q   E   R   Y     126
CAC GGT GTG CTG GAT GCA ACT CGA CTG CCG GCA ACT TGT GTG CAA GAA AGA TAT     378
    T73                                   T84

 E   Y   F   P   G   F   S   G   E   E   I   W   N   P   N   T   N   V     144
GAA TAT TTT CCT GGT TTC TCA GGC GAA GAG ATA TGG AAT CCA AAT ACA AAC GTT     432
        P94

 S   E   D   C   L   Y   I   N   V   W   A   P   A   K   A   R   L   R     162
TCA GAG GAT TGC TTG TAC ATT AAT GTT TGG GCA CCA GCG AAA GCG CGT TTA AGG     486

 H   G   R   G   A   N   G   G   E   H   S   N   K   A   D   T   D   H     180
CAT GGA CGC GGC GCT AAT GGC GGT GAG CAC TCC AAT AAA GCC GAC ACC GAT CAT     540

 L   I   H   N   G   N   P   Q   N  T/A  T   N   G   L   P   V   L   I     198
TTG ATA CAT AAC GGA AAT CCG CAA AAC RCC ACA AAC GGC TTA CCC GTG CTT ATT     594
    H116                                                 I130

 W   I   Y   G   G   G   F   M   T   G   T   A   T   L   D   I   Y   N     216
TGG ATT TAT GGT GGT GGC TTT ATG ACC GGC ACT GCC ACA TTG GAC ATT TAC AAT     648

 A   D   I   M   S   A   V   G   N   V   I   V   A   S   F   Q   Y   R     234
GCG GAC ATT ATG TCC GCT GTG GGT AAT GTA ATA GTG GCT TCA TTT CAA TAT CGT     702

 V   G   A   F   G   F   L   H   L   S   P   A   M   P   G   Y   E   E     252
GTT GGC GCA TTT GGC TTC CTA CAT TTA TCG CCC GCC ATG CCT GGC TAT GAG GAG     756

 E   A   P   G   N   V   G   L   W   D   Q   A   L   A   I   R   W   L     270
GAG GCG CCC GGT AAT GTT GGC TTG TGG GAT CAA GCA TTG GCC ATA CGT TGG TTG     810

 K   T   N   A   H   A   F   G   G   N   P   E   W   M   T   L   F   G     288
AAA ACG AAT GCA CAT GCC TTT GGC GGT AAT CCG GAG TGG ATG ACA CTG TTT GGT     864
    T280                                                            G

 E   S   A   G   S   S   S   V   N   A   Q   L   V   S   P   V   T   A     306
GAA TCG GCT GGT TCG AGT TCG GTG AAT GCG CAG TTG GTG TCG CCA GTG ACG GCG     918
                                                          C333

 G   L   V   K   R   G   M   M   Q   S   G   T   M   N   A   P   W   S     324
GGT CTG GTG AAG CGT GGT ATG ATG CAA TCG GGC ACA ATG AAT GCG CCA TGG AGT     972

 H   M   T   S   E   K   A   V   E   I   G   K   A   L   I   N   D   C     342
CAT ATG ACG TCA GAG AAG GCA GTA GAA ATC GGC AAA GCC TTA ATC AAT GAT TGC    1026
    I234                                                            C265

 N   C   N   A   S   L   L   A   E  N/D  P   Q   A   V   M   A   C   M     360
AAT TGC AAT GCG TCA CTG TTG GCG GAA RAT CCT CAA GCT GTA ATG GCT TGC ATG    1080
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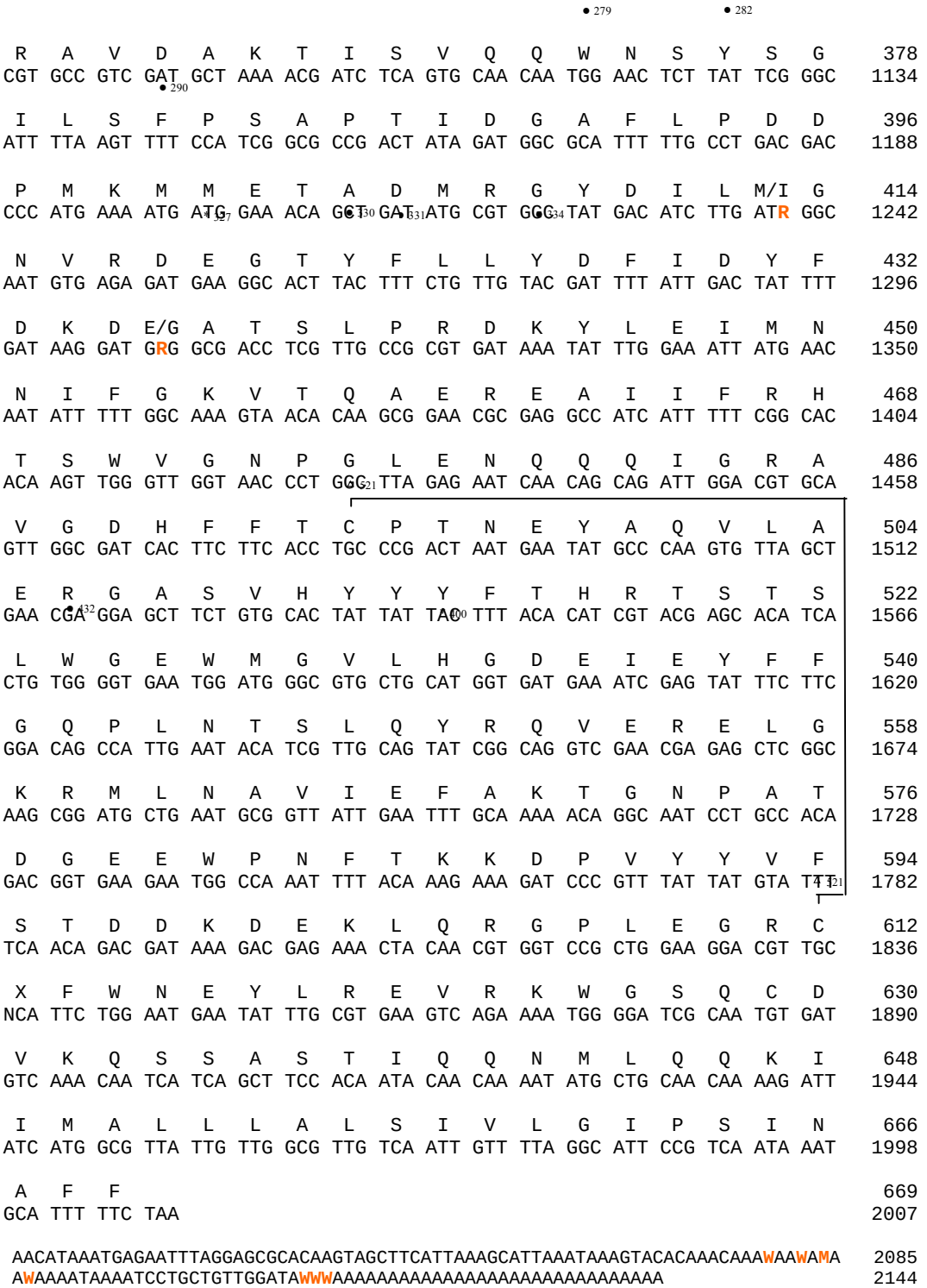

Figure 3. Alignment of AChE protein and nucleotide sequence of single individuals from the C strain of *C. capitata* obtained from RT-PCR and direct sequencing (below the complete nucleotide sequence and above the inferred amino acid sequence). In bold orange are indicated the polymorphisms found among the sequence, which are indicated using the one-letter IUPAC-IUB code [R (A,G); M (A,C) and Y (C,T)]. The three residues composing the catalytic triad (Ser200, Glu327 and His440) are indicated by (*) (the aminoacid numering is that of *Torpedo*). The filled circles represent 13 aromatic residues lining the active gorge and the open circles the residues (Gly118, Gly119 and Ala201) that form the oxy-anion hole. The conserved Gly-Xaa-Ser-Xaa-Gly

3.1.3 Point mutations of AChE in W resistant strain

The existence of mutations in the *ace* gene associated to a reduction in sensitivity to malaoxon in the WR phenotype compared to the WS and C phenotypes was investigated. A fragment of 1907 nucleotides (-81 to 1826) of *Ccace* cDNA from the thorax of three individuals of each phenotype was amplified by RT-PCR and direct sequencing using specific primers (Face_4 and Race_3, Table 1).

When the cDNA sequences of the AChE from individuals of the WR and C phenotypes were aligned, a non synonymous single nucleotide substitution GGC to GCC was found in all individuals analyzed from the W phenotype. This change in the codon was always in homozygosis and represents an amino acid substitution of glycine to alanine at residue 462 in the predicted AChE protein. In the case of the WS phenotype, two of the three individuals analyzed presented this mutation in heterozygosis and the third individual did not present the mutation.

3.1.4 Quantitative analysis of the transcript of *Ccace* gene

QPCR was used to know if there was a different level of the expression of AChE between individuals of the W strain that survived a concentration of 3000 ppm of malathion in the diet for 48 hours and individuals from the C strain. No significant differences (Mann-Whitney test, $p>0.05$) in AChE expression were found between both W and C strains.

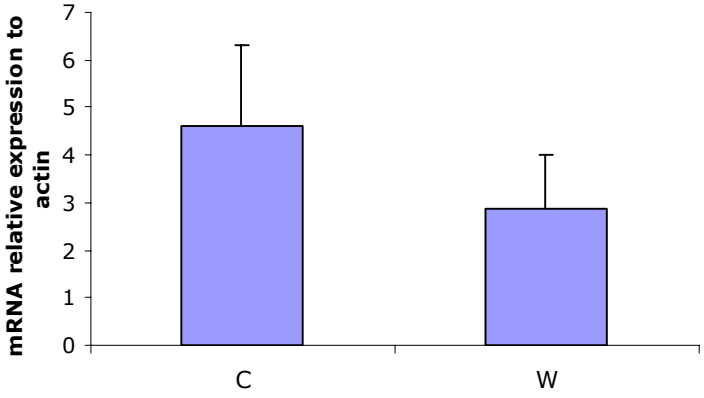

Figure 4. AChE mRNA levels in 8 individual from C and W strains of *C. capitata.* Data represented are the ratio of AChE to actin ± standard error.

3.3 Discussion

Biochemical studies performed in chapter 2, using pools of head adult Medflies from the susceptible C and the resistant W strains, indicated that target site (AChE) insensitivity may be involved in the resistance of *C. capitata* to malathion. In the present study, we have shown that AChE was susceptible to malaoxon (10^{-7} M) in all individuals from the C strain, whereas two different phenotypes were distinguished in the W strain: individuals (WS) with the same phenotype as individuals from the C strain, and individuals (WR) with an insensitive phenotype (reduced AChE activity and less sensitivity to inhibition by malaoxon). These results are in concordance with the lower slope value obtained in the malathion susceptibility assay of the W strain (0.9) in comparison with the laboratory C strain (2.3) (Chapter 2), indicating more heterogeneity within the resistant population.

The full-length AchE cDNA from the susceptible strain (C) of *C. capitata* has been obtained. Phylogenetic analysis showed that the *Ccace* gene described here may be designated as an *ace2* homolog according with the nomenclature of Weill *et al*., (2003). The deduced AChE protein is 93% identical to that of *Bactrocera dorsalis* (Hsu, *et al.*, 2006), 93% identical to the sequence of *Bactrocera oleae* (Vontas *et al.*, 2002a) and 74% identical to the sequence of *D. melanogaster* (Hall and Spierer, 1986). The putative AChE protein (Figure 3) has all of the features characteristic common to other acetylcholinesterases, namely: the catalytic triad (Ser200, Glu327 and His440, the amino acid numbering is that of Torpedo); the pentapeptide "Gly-Xaa-Ser-Xaa-Gly" motif (around position Ser200), characteristic of the active site of cholinesterases (Oakeshott *et al.*, 1993); the six cysteines involved in three conserved disulphide bonds, and the residues that form the oxy-anion hole, that helps to stabilize the tetrahedral molecule during catalysis (Soreq *et al.*, 1992). The active site gorge is coated with 13 aromatic side chains as in *D. melanogaster*, including the choline binding site (Sussman *et al*., 1991; Harel *et al.*, 2000). Some polymorphism was found in the clones analyzed, but they were not present in the sequences of the single individuals.

A total of 14 mutations have been reported in AChE associated to resistance to insecticides in higher Diptera (Mutero *et al.*, 1994; Kozaki *et al.*, 2001; Vontas *et al.*, 2001, 2002a; Walsh *et al.*, 2001; Hsu *et al.*, 2006). To investigate whether these or other mutations were present in individuals of the resistant strain, the *Ccace* gene was

generated by RT-PCR from individuals of the three phenotypes investigated, WR, WS and C. Sequence analysis showed an amino acid substitution, glycine for alanine, at position 462 of the *Ccace* gene (368 and 328 in *Drosophila* and *Torpedo* numbering, respectively), that was found in homozygosis in individuals with the WR phenotype, in heterozygosis or absent in individuals with the WS phenotype, and absent in individuals from the susceptible strain. This mutation is adjacent to the glutamate of the catalytic triad in *D. melanogaster* (Walsh *et al.*, 2001), and has been associated with insecticide resistance in populations of *D. melanogaster*, *Musca domestica* and *Plutella xylostella* (Walsh *et al.*, 2001; Menozzi *et al.*, 2004; Baek *et al.*, 2005; Lee *et al*., 2007). Our results showed that this mutation in homozygosis (individuals of the resistant W strain with the WR phenotype) conferred insensitivity to malaoxon, but not to the other oxon OP insecticides assayed. Site-directed mutagenesis of Gly328Ala (*Torpedo* numbering) in *D. melanogaster* and *M. domestica* led to a decreased susceptibility to insecticides, affecting mainly the insensitivity to malaoxon (Walsh *et al.*, 2001; Menozzi *et al.*, 2004). The AChE of the individuals with the WR phenotype showed higher k_m (lower affinity) and lower V_{max}, resulting in a 2.4-fold reduction in the efficiency (V_{max}/k_m) of the altered enzyme. Similar reductions in the affinity for the substrate have been obtained in resistant strains of *M. domestica* and *P. xylostella* containing this mutation (Walsh *et al.*, 2001; Lee *et al.*, 2007). With respect to the individuals of the resistant strain that present the mutation in heterozygosis (WS phenotype), the AChE was sensitive to malaoxon and their activity was not altered. This may indicate that the resistance is recessive or that other resistance mechanisms are involved. Nevertheless, the susceptibility of WS individuals to malathion by ingestion must be tested, since there is not always a correspondence between in vivo and in vitro assays. Ongoing studies suggest that the inheritance of resistance to malathion in *C. capitata* is co-dominant, as has being reported for other cases of resistance to OPs insecticides mediated by modifications at the target site (Bourguet *et al.*, 1996b).

Changes in the regulation of the AChE may also play a role in the resistance to OPs. Charpentier and Fournier (2001) showed that there was a correlation in natural populations of *D. melanogaster* between the amount of AChE in the central nervous system and their susceptibility to insecticides. The results obtained here showed no significant differences in AChE expression among in individuals from the W resistant strain compared to the susceptible strain, indicating that resistant to malathion in *C. capitata* is not achieved by an up regulation of the enzyme.

In conclusion, a point mutation Gly328Ala (*Torpedo* numbering) in the AChE cDNA has been associated with resistance to malathion in *C. capitata*. Adult flies from the resistant W strain that present this mutation in homozygosis were less sensitive to inhibition by malaoxon suggesting that target site insensitivity allows them to survive to high concentrations of the insecticide. Nevertheless, other resistant mechanism, such as metabolic resistance, may also play a role in the resistance of *C. capitata* to malathion.

4 Metabolic resistance to malathion in *C. capitata*

Resistance to malathion has been detected in Spanish field populations of the Mediterranean fruit fly (Medfly), Ceratitis capitata (Wiedemann) (Diptera: Tephritidae) (Chapter 2). A resistant field-derived strain (W) and a susceptible laboratory strain (C) are being investigated to determine the nature of the resistance. A point mutation Gly328Ala (Torpedo numbering) in the acetylcholinesterase (AChE) gene, that makes the enzyme less sensitive to malathion, was associated with resistance in adult flies of the W strain (Chapter 3). Besides, the esterase inhibitor DEF enhanced the toxicity of malathion in the W strain (Chapter 2), suggesting that metabolic resistance mediated by esterases may also be involved in the loss of susceptibility to malathion.

Resistance to malathion may be achieved by enhancing activity of metabolic systems like cytochrome P450 (Weeling et al., 1974; Morton and Holwerda, 1985; Maitra et al., 2000), glutathione S-transferases (Wool et al., 1982; Taskin and Kence, 2004) and esterases (Campbel et al., 1998a; Karunaratne and Hemingway, 2001). Increase of esterase activity, due to an amplification of esterase genes, confers resistance to a broad spectrum of organophophate (OP) insecticides. The overproduced esterases sequester and very slowly hydrolyze OPs, preventing inhibition of the AChE target site (Hemingway and Karunamaratne, 1998; Blackman et al., 1999). Specific resistance to malathion has been attributed to carboxylesterases which catalyze the carboxyl esters of malathion, normally referred to as malathion carboxylesterases (MCE) (Hughes et. al., 1984; Hemingway, 1985). The increase in MCE activity has been associated with a decrease in general esterase activity in higher Diptera, such as Musca domestica (van Asperen and Oppennoorth, 1959) Lucilia cuprina (Hughes and Raftos, 1985) and Chrysomya putoria (Townsend and Busvine, 1969). This has been explained by the mutant ali-esterase hypothesis (Oppenoorth and van Asperen, 1960; Bigley and Plapp, 1960). This hypothesis proposes that a structural mutation in a carboxylesterase (αE7) results in a reduced ability to hydrolyze carboxylesterase substrates, but an acquired ability to hydrolyze OP substrates. Two amino acid substitutions (Gly137Asp and Trp251Ser/Leu Drosophila numbering) have been described in M. domestica and L. cuprina that confer resistance to OPs: Gly137Asp confers the "diazinon" type of OP

resistance and Trp251Ser/Leu substitutions confer "malathion" type OP resistance (Campbell et al., 1998a,b; Claudianos et al., 1999; Taskin and Kence, 2004; Taskin et al., 2004).

In this study, we determine the general detoxification potential of the susceptible C and the resistant W strains by measuring esterases, cytochrome P450 monooxygenase and glutathione S-tranferase activities. The role of ali-esterases in the resistance of C. capitata to malathion has been further investigated by assessing its substrate specificity and sequencing the αE7 gene of C. capitata.

4.1 Materials and methods

4.1.1 Insects

The study was performed using Medflies from the susceptible (C) and the resistant (W) strains described in section 2.1.1. The flies from the W strain were subjected to 3000 ppm of malathion for 24-48 hours in order to obtain about 50 % of the mortality. Alive Medflies were frozen in liquid nitrogen and stored at -80ºC until needed.

4.1.2 Enzymatic assays

Except as otherwise stated, the products were purchased from Sigma Chemical Co.

Preparations of tissue homogenate: Adults and third instars larvae from the C strain were used for the enzymatic characterization of general esterases, P450 and glutathione S-transferases enzymes. The adults were dissected and gut (midgut and crop), fat body and thorax removed and homogenized in 0.15 M NaCl (one crop in 50 µl, one midgut in 50 µl, one thorax in 100 µl and one fat body in 100 µl). Third instars larvae were dissected and midguts homogenized in of 0.15 M NaCl (1 midgut in 100 µl). The tissues from 100 individuals were pooled, centrifuged at 12000 rpm for 5 min in a UNIVERSAL 32 R (Hettich) centrifuge at 4ºC, and the supernatant collected and stored at -20ºC until needed. For the comparison of enzymatic activities between individuals from the C and W strains, abdomens from single adults (3-5 days old) were homogenized in 0.15 M NaCl (one abdomen in 200 µl). Solubilized protein was isolated

as described above. The protein concentration was determined according to the procedure of Bradford (1976) as described in section 2.1.3.

Enzymatic activity: Except as otherwise stated, all assays were carried out in triplicate and blanks were used to account for spontaneous breakdown of substrate in 100 µl of reaction mixture. Reaction buffers were: 0.1 M citrate (pH 6.0-7.0); 0.1 M Tris-HCl (pH 6.0-10.0) and 0.1 M potassium phosphate (pH 6.0-8.0) (kPB). All buffers contained 0.15 M NaCl and 5 mM MgCl2. All activities were performed at their optimum pH. The absorbance values were determined using either a Molecular device Versamax microplate reader or Hitachi U-2000 spectrophotometer.

Esterases: General esterase activity was assayed using the substrates α- or β-naphthyl acetate (α-Na and β-Na, respectively) by the spectrophotometric method of Dary et al. (1990). The reaction mixture (167 µl) contained 3 µl of extract, 0.25 mM α- or β-Na (added in 0.1% of EtOH), and 0.1 M kPB at pH 7.5. The mixture was incubated at 30ºC for 30 min, and the reaction was terminated by the addition of 84 µl of a reagent solution (3 mg fast garnet salt and 15 mg of sodium dodecyl sulfate (SDS) per ml of water). The solution was incubated for 30 min at room temperature and the absorbance was determined at 527 and 505 nm for α- or β-naphthol product, respectively. The activity was expressed as nanomoles of substrate hydrolyzed per min and per mg of protein, using α- or β-naphthol as standards.

Ali-esterase activity was measured using S-methyl thiobutanoate (MtB) as the substrate. The reaction mixture (200 µl) contained 75 µl of extract, 2 mM MtB, 10 mM DTNB (Ellman's reagent), and 0.1 M kPB at pH 8.0. The change in absorbance at 412 nm was measured for a period of 5 min. The ali-esterase activity was converted to nanomoles of substrate hydrolyzed per min and per mg of protein, using the extinction coefficient of the Ellman's product (Є412= 1.36x104 M-1 cm-1) (Ellman, 1959).

Activity against 4-methyl umbellyferyl acaetate (4-MUA, Fluka S.A.), 4-methyl umbelliferyl butanohate (4-MUB, Fluka S.A.), 4-methyl umbellifery heptanoate (4-MUH, Fluka S.A.) and 4-methylumbelliferyl phosphate (4-MUP, Fluka S.A.) were determined with a Cary Eclipse Fluorescece spectrofluorometer (Varian) with excitation at 365 nm and emission at 445 nm. Stock solutions of 4-MUA, 4-MUB, 4-MUH and 4-MUP were prepared by dissolving the substrates at 1.0 mM in dimethyl sulfoxide. The substrates where diluted into reaction buffer (0.1 M Tris-HCl buffer, pH 8) immediately prior to performing the experiment. The reaction mixture (200 µl)

contained 2.5 µl extract, 0.25 mM of the corresponding substrate, and 0.1 M Tris-HCl at pH 8. The reaction mixture was incubated at 30ºC for 5 min and the increment of fluorescence was measured for 5 min. The activity was expressed as nanomoles of substrate hydrolyzed per min and per mg of protein, using 4-methylumbelliferone (4-MU, Fluka S.A.) as standard.

Malathion carboxylesterase activity (MCE) was measured readiometrically by modifications of the procedures of Whyard et al. (1994) using [14C] malathion as substrate. [14C] radiolabeled malathion, with the label in the two bridge carbon atoms of the succinate moiety of the molecule, and had an specific activity of 80 mCi/mmol. It was diluted with toluene to give a working solution with an activity of 10 µCi/ml. Reaction mixture (103 µl) contained 100 µl of extract, 2.5 µM of the solution of [14C] malathion and 20 µM of unlabeled malathion. The mixture was incubated at 30ºC for 1 hour. Thereafter, the unhydrolized malathion was separated from the malathion monoacids by chloroform extraction: 1 ml of chloroform was added to the mixture, mixed, and the phases separated by centrifuging at 15000 g for five min. The aqueous phase was diluted into ReadySafe scintillation fluid and counted in a Wallac liquid scintillation counter (Pharmacia) to determine the metabolites produced by the hydrolysis of [14C] malathion. Incubations without enzyme were also performed as a control for non-enzymatic hydrolysis of malathion.

Cytochome (P450) monooxygenases: P450 monooxygenases were assayed by the reduction of cytochrome c by NADPH-cytochrome P-450 reductase according to Masters et al. (1965). The reaction mixture (100 µl) contained 5 µl of midgut extract, 50 µM cytochrome c, NADPH-generating system [0.5 mM NADP (nicotinamide adenine dinucleotide phosphate); 2.5 mM glucose-6-phosphate; and 0.25 units of glucose-6-phosphate dehydrogenase], and 0.1 M Tris-HCl buffer pH 7.0. The mixture was incubated at 30ºC for 4 hours, thereafter the reaction was stopped adding 50 µl of methanol, centrifuged at 10000 g for 5 min and the absorbance of the supernatant was determined at 550 nm. The activity was expressed as nanomoles of substrate reduced/min/mg protein, using a molar extinction coefficient of 27.6 mM-1 cm-1 for the reduced form of cytochrome c (Margoliash and Frohwirt, 1959)

Glutathione S-transferases: Glutathione S-transferase activity was measured in 100 µl of reaction mixture containing 20 µl of midgut extract, 1 mM CDNB (1-chloro-2,4-dinitrobezene), 3 mM reduced glutathione, and 0.1 M Tris-HCl buffer at pH 8.0. The buffer containing the extract and the reduced glutathione was incubated at 30ºC

for 15 min, and the reaction started by the addition of the substrate. The increment in absorbance at 340 nm was recorded during 2 min to determine the nanomoles substrate conjugated/min/mg protein, using a molar extinction coefficient of 9.6 mM-1 cm-1 (Habig et al., 1974).

4.1.3 Cloning and sequencing of *αE7* cDNA from *C. capitata*

Total RNA was extracted from 100 gut adults (3-5 day old) with TRIzol® reagent, as described in section 3.1.3. Poly(A)+ RNA was further purified by using mRNA purification kit (Amershan Pharmacia Biotech. Ltd.). A reverse transcription and adaptor-ligation PCR was performed on Poly(A)+ RNA using Marathon cDNA amplification Kit (Clontech Laboratories Inc.) following the manufactured instructions. An internal cDNA fragment (483 pb) was amplified from the first strand cDNA through a set of degenerate oligonucleotides dFaE7 and dRaE7 (Table 1) designed based on a aligned of Drosophila melanogaster (Accession no. NP_524261), L. cuprina (Accession no. AAB67728), M. domestica (Accession no. AAD29685) and Haematobia irritans (Accession no. AAF14517) αE7 amino acid sequences. PCR was conducted in a DNA thermal cycler (model 2400, Perkin Elmer). The 25 µl PCR reaction contained 2 µl of marathon cDNA, 0.4 µM of each primer, 3 mM MgCl2, 0.2 µM of each dNTP and 4 units of AmpliTaq® DNA polymerase (Roche) in the manufactures's reaction buffer. Amplification began with an initial desnaturation step of 1 min at 94ºC; followed by 35 cycles of 1 min at 94º, 1 min at 50ºC and 2 min at 72ºC; and final step of 7 min at 72ºC to fully extend all PCR products.

For the amplification of cDNA ends, specific primers were designed from the fragment sequenced. The 5' end was amplified using Marathon cDNA as a template by PCR and the Marathon adaptor and the specific RiaE7_Cc1 primers (Table 1). The 25 µl PCR reaction contained 0.1 µM of each primer, 0.2 mM of each dNTP and 0.5 µl of 50X BDTM 2 Advantage Polymerase mix (BD AdvantageTM 2 PCR kit, Clontech) in the manufacturer's reaction buffer. The PCR conditions for the reaction were as follow: an initial desnaturation at 94ºC for 2 min; followed by 35 cycles consisting of 30 sec at 92ºC, one min at 50ºC and 1.5 min at 72ºC; and a final step of 7 min at 72ºC. The 3' end was obtained using the Marathon cDNA as a template by PCR with primers, FiaE7_Cc3 and Marathon adaptor (Table 1). The 25 µl PCR reaction contained 0.1 µM of each primer, 0.2 mM of each dNTP and 0.5 µl of 50X BDTM 2 Advantage Polymerase mix (BD AdvantageTM 2 PCR kit, Clontech) in the manufacturer's reaction

buffer. The PCR conditions were as follows: an initial denaturation at 94ºC for 2 min; followed by 35 cycles consisting of 30 sec at 92ºC, one min at 55ºC and 1.5 min at 72ºC; and a final step of 7 min at 72ºC.

PCR products were purified, cloned, sequenced and analyzed as described in section 3.1.3.

4.1.4 RT-PCR and direct Sequencing of *αE7* cDNA from single individuals

Total RNA was extracted from the thorax of individual flies with TRIzol® reagent (Molecular Research Center), and used as template for the first strand cDNA synthesis. cDNA synthesis was performed with Reverse Transcriptase AMV (Roche) and with AnchorT as a primer (Table 1). A first PCR reaction was performed using Achor and F0aE7_Cc (Table 1) as primers and a second nested PCR was carried out using specific primers F0iaE7_Cc and R0aE7_Cc (Table 1). In both cases the 25 µl PCR reaction contained 0.1 µM of each primer, 0.2 mM of each dNTP and 4 units of AmpliTaq Gold® Taq (Roche) in the manufacturer's reaction buffer. The PCR conditions for both reactions were as follows: an initial denaturation at 95ºC for 15 min followed by 35 cycles consisting of 30 sec at 94ºC, 30 sec at 55ºC and 1 min at 72ºC; and a final step of 5 min at 72ºC was used to fully extend all PCR products.

Table 1. Primers designed to amplify *αE7* gene of *C. capitata*.

Primer[a]	Nucleotide sequence 5'-3'
dFiaE7	GAGGA[CT]TG[CT][CT]T[AC]TA[CT][CT]T[GCA]A[AG]TGT
dRiaE7	TC[AG]TT[AG]T[CT][CT]TC[GTA]CC[CT]TT[AG]TA[AG]CC
FiaE7_CcE1	TTGTGCGAGATTCGGTGGCAACCC
RiaE7_CcE1	CGACATTAGAATGGCGCGATGGAAAAGAC
FiaE7_CcE3	TGAAGACTGTCTCTACCGCAATGTGT
RiaE7_CcE3	CACCCTTATATCCAGCGTATTTTGC
F0iaE7_Cc	CAAATACCAACAAAATCGACTGGCCAC
F0aE7_Cc	CCGTTGGCGTTTAAAAGTCTACGAACA
R0aE7_Cc	GCCCACACCTTCGCTTTGGCCAATTC
FqaE7_Cc	CAAGCATAGCCCAGAGACAGA
RqaE7_Cc	GGAGTTTTGCCGCTCACA
Anchor	GCCACGCGTCGACTAGTACG
AnchorT	GCCACGCGTCGACTAGTACGTTTTTTTTTTTTTTTTTTTTTTT

[a] The suffixed d, F and R in primers names indicate degenerate, forward and reverse, respectively.

4.1.5 Quantitative Real-Time PCR (QPCR)

QPCR has been carried out as has been explained in 3.1.5. In this case, the primers used for αE7 gene were FqaE7_Cc and RqaE7_Cc (Table 1).

4.2 Results

4.2.1 Detoxification enzyme activities in the susceptible (C) and resistant (W) strains

The main detoxification enzyme systems in adults and larvae of C. capitata were characterized using the C strain. Table 2 shows the specific activities of these enzyme systems, in different adult tissues and in larval midgut. The highest specific activities, for all these enzymes, were obtained in the adult midgut. Independently of the tissue, the hydrolysis of the general esterase substrate, α-Na presented a maximum activity

at pH7.5. Likewise, the optimum pHs for P450 and GST activities were 7 and 8, respectively.

Table 1. Detoxification enzyme activities in different tissues in adults and larvae from the susceptible C strain of *C. capitata.*

Enzyme[a]	pH	Specific activity[b]				
		Larvae	Adults			
		midgut	Gut		fat body	thorax
			midgut	crop		
EST	7.5	82.4 ± 12	133.3 ± 8.4	87.7 ± 8.3	4.5 ± 0.4	9.9 ± 0.2
P450	7.0	8.9 ± 0.7	20.8 ± 0.2	7.6 ± 0.5	5.9 ± 0.04	1.3 ± 0.1
GST	8.0	78.0 ± 8.1	128.5 ± 5.0	67.4 ± 8.2	51.2 ± 1.6	29.3 ± 0.6

[a] EST, esterase activity using α-naphtyl acaetate (α-Na) as a substrate; P450, cytochrome P450 using cytochrome c as substrate and GST, glutathione S-transferase using CDNB as substrate.

[b] Pools of midgut extract from larvae and from different tissues of adults from the C strain were used. Values are mean ± standard error of three experimental replicates. Specific activity was measure as nanomoles of α-Na hydrolyzed/min/mg protein, nanomoles of citochrome c reduced/min/mg protein or nanomoles of CDNB conjugated/min/mg protein for esterases, P450 or GST, respectively.

Specific activities of the detoxification enzymes in both, the C and W strains were compared using the abdomen of single adults (Table 3). The specific activities of cytochrome P450 activity, determined by cytochrome c reduction, and GST activity, determined using CDNB conjugation, were no different in the W and C strains. Likewise, no significant differences were found when esterase activity was measured using α-Na, β-Na, MUA or MUP as substrates. However, esterase activity determined by MUH and MUB hydrolysis was 2.5-fold higher and 2.4-fold lower, respectively, in the W strain. More importantly, a reduction of 1.4-fold in the hydrolysis of MtB, substrate used to measure the ali-esterase activity, was found in the W strain compared to the C strain.

Table 2. Specific activity of the detoxification enzymes in the susceptible C and the resistant W strains of *C. capitata*.

Enzyme[a]	Substrate	Specific activity[b]	
		C	W
P450	Cirochrome c	5.4 ± 0.5	4.2 ± 0.5
GST	CDNB	288.2 ± 13.9	288.9 ± 40.2
EST	α-Na	290. 2± 27.3	273.6 ± 29.2
	β-Na	298.9 ± 23.3	259.2 ± 34.2
	MUA	3.0 ± 0.2	3.2 ± 0.2
	MUB	2.9 ± 0.2	2.0 ± 0.2*
	MUH	3.2 ± 0.3	5.0 ± 0.4*
	MUP	0.07 ± 0.01	0.07 ± 0.01
	MtB	20.5 ± 2.0	12.4 ± 1.0*

[a] P450, cytochrome P450 using cytochrome c as substrate; GST, glutathione S-transferase using CDNB as substrate; EST, esterases using α-naphtyl acetate (α-Na) and β-naphtyl acetate (β-Na), methyl umbelliferyl acetate (MUA), methyl umbelliferyl butanoate (MUB), methyl umbelliferyl heptanoate (MUH), methyl umbelliferyl phosphate (MUP) and S-methyl thiobutanoate (MtB) as substrates.

[b] Values are mean of the specific activity ± standard error of 10-25 individuals of each strain. Specific activity was measure as nanomoles of α-Na, β-Na, MUA, MUB, MUH, MUP or MtB hydrolyzed/min/mg protein; nanomoles of citochrome c reduced/min/mg protein; nanomoles of CDNB conjugated/min/mg protein.

*Mean of the W strain was significantly different from the C strain ($p<0.05$, Mann-Whitney test)

In vitro hydrolysis of malathion, using [14C] malathion as substrate, was assayed in 10 individuals of each strain to determine if specific malathion carboxylesterases (MCE) were involved in this resistance. The results obtained showed similar and very low levels of malathion hydrolysis for the W (0.43 ± 0.05 pmol/min/mg of protein) and C (0.31 ± 0.04 pmol/min/mg of protein) strains.

4.2.2 Sequencing of *αE7* cDNA from *C. capitata*

Aligned D. melanogaster, L. cuprina, M. domestica and H. irritans αE7 amino acid sequences were used to construct two degenerate PCR primers (dFiαE7 and dRiαE7, Table 1) to sequence a region of this gene in C. capitata. PCR from the marathon cDNA library prepared from gut of the C strain yielded 15 amplicons of 483 pb (position from 319 to 802, Figure 1) (Data not shown). Sequencing of these amplicons

showed to be fragments of various members of the α-esterase gene cluster (Data no showed). Three of these amplicons showed high nucleotide sequence similarity with αE7 from other higher Diptera. Then, specific primers were designed from this fragment two amplify the full length cDNA of αE7. Three positive clones from each end (5' and 3') were completely sequenced to obtain the full-length of cDNA (1707 pb) αE7 from C. capitata (CcαE7) (Figure 1).

```
GACATT                                                                -6

 M   Q   S   N   I   G   F   I   E   K   F   R   W   R   L   K   V   Y    18
ATG CAG TCA AAT ATT GGA TTT ATT GAA AAA TTC CGT TGG CGT TTA AAA GTC TAC    54

 E   H   K   Y   Q   Q   N   R   L   A   T   A   E   T   L   I   V   E    36
GAA CAC AAA TAC CAA CAA AAT CGA CTG GCC ACT GCA GAA ACG TTA ATT GTA GAG   108

 T   E   Y   G   K   V   E   G   I   K   R   L   S   I   Y   N   I   P    54
ACT GAA TAT GGA AAA GTA GAA GGC ATT AAA CGC TTA AGT ATT TAC AAC ATT CCT   162

 Y   Y   S   F   E   G   I   P   Y   A   Q   P   P   V   G   E   L   R    72
TAC TAC AGC TTC GAG GGT ATA CCT TAT GCC CAA CCA CCT GTG GGT GAG CTA CGC   216

 F   R   A   P   Q   R   P   T   P   W   E   G   V   R   D   C   K   S    90
TTC AGA GCA CCT CAA AGG CCA ACT CCA TGG GAG GGT GTG CGA GAT TGC AAA AGC   270

 T   K   E   M   A   V   Q   T   H   I   I   T   G   I   L   E   G   S   108
ACC AAA GAA ATG GCG GTA CAA ACA CAT ATC ATA ACT GGA ATA CTG GAA GGA TCT   324

 E   D   C   L   Y   L   N   V   Y   T   N   N   T   L   P   D   K   P   126
GAA GAC TGT CTC TAC CTC AAT GTG TAT ACG AAT AAT ACT CTG CCT GAT AAG CCG   378

 R   P   V   M   I   W   I   H   G   G   G   L   C   T   G   E   A   T   144
CGC CCA GTT ATG ATA TGG ATA CAT GGT GGT GGA CTT TGT ACT GGA GAG GCG ACA   432

 R   E   W   Y   G   P   D   Y   F   M   Q   K   D   I   V   L   V   T   162
CGT GAA TGG TAT GGA CCT GAT TAT TTC ATG CAA AAA GAT ATT GTG CTT GTG ACA   486

 M   Q   Y   R   L   G   V   L   G   F   L   S   L   G   T   P   E   L   180
ATG CAA TAT CGG CTA GGA GTA TTG GGC TTC CTT TCG CTG GGC ACA CCC GAA CTC   540

 N   V   P   G   N   S   G   L   K   D   Q   V   L   A   I   K   W   V   198
AAC GTA CCT GGA AAC TCT GGT CTG AAA GAC CAA GTA TTG GCT ATA AAA TGG GTG   594

 K   N   N   C   A   R   F   G   G   N   P   D   C   I   T   V   F   G   216
AAA AAT AAT TGT GCA AGA TTC GGT GGC AAC CCC GAC TGC ATA ACT GTA TTC GGT   648

 E   S   A   G   A   T   S   A   H   C   M   M   L   T   E   Q   T   Q   234
GAA AGT GCT GGT GCG ACG TCT GCG CAT TGT ATG ATG CTC ACT GAA CAG ACA CAA   702

 G   L   F   H   R   A   I   L   M   S   G   T   A   L   P   L   W   E   252
GGT CTT TTC CAT CGC GCC ATT CTA ATG TCG GGT ACG GCG CTA CCC CTA TGG GAG   756

 T   E   D   Q   K   L   R   A   F   D   L   A   K   Y   A   G   Y   K   270
ACA GAG GAT CAA AAA TTA CGT GCT TTC GAT CTC GCA AAA TAC GCT GGA TAT AAG   810

 G   V   D   N   D   K   D   V   L   A   Y   L   R   K   C   K   A   K   288
GGT GTC GAT AAC GAT AAG GAT GTG TTG GCA TAT TTG CGC AAG TGC AAA GCG AAA   864

 D   L   I   A   L   E   G   R   T   L   T   A   E   D   R   A   R   N   306
GAT TTA ATT GCG CTC GAA GGT CGT ACA CTT ACT GCG GAG GAT CGT GCA CGT AAC   918
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 I   S   T   P   F   V   Y   C   V   E   P   Y   V   T   P   E   C   V     324
ATA TCT ACG CCA TTC GTA TAT TGT GTA GAA CCG TAT GTG ACA CCT GAA TGT GTT    972

 I   Q   K   P   I   R   E   M   M   R   T   A   W   G   N   A   I   P     342
ATA CAA AAG CCG ATA AGG GAA ATG ATG AGA ACA GCG TGG GGT AAT GCG ATA CCG   1026

 L   L   V   G   H   A   S   D   E   G   L   I   F   L   Q   G   A   K     360
TTA TTA GTT GGT CAT GCG TCA GAT GAG GGG CTG ATC TTC TTG CAA GGC GCT AAG   1080

 I   L   A   S   I   A   Q   R   Q   K   S   Y   S   L   K   P   F   V     378
ATT TTA GCA AGC ATA GCC CAG AGA CAG AAA AGT TAT TCA TTA AAA CCA TTT GTA   1134

 P   Y   E   V   A   D   S   E   D   N   E   K   F   E   Q   K   L   R     396
CCT TAT GAA GTG GCG GAC AGC GAA GAT AAT GAA AAA TTT GAA CAG AAA CTG AGA   1188

 T   S   H   V   S   G   K   T   P   T   V   E   E   F   K   N   I   I     414
ACG TCG CAT GTG AGC GGC AAA ACT CCA ACA GTT GAG GAA TTC AAA AAT ATC ATC   1242

 A   Y   A   Y   L   H   F   P   L   Y   R   L   I   R   S   R   L   T     432
GCC TAT GCA TAT CTG CAC TTT CCA CTC TAC CGA CTA ATA CGC TCG CGC TTG ACA   1296

 Y   A   A   G   A   P   L   Y   L   Y   R   F   D   F   D   S   E   E     450
TAT GCG GCT GGC GCG CCC CTC TAC CTA TAT CGC TTC GAT TTT GAT TCC GAA GAA   1350

 L   P   H   P   Y   R   I   L   R   N   G   R   G   V   K   G   V   A     468
TTG CCA CAT CCC TAT CGT ATT CTA CGC AAT GGC CGT GGT GTA AAA GGC GTA GCG   1404

 H   G   D   E   L   S   Y   I   F   T   N   L   F   S   C   T   L   S     486
CAT GGC GAT GAA CTT TCC TAT ATA TTT ACA AAC CTA TTT TCC TGC ACA TTA TCC   1458

 K   E   S   R   E   Y   R   T   I   E   R   M   V   G   F   W   T   Q     504
AAA GAG AGT CGT GAG TAT CGC ACC ATT GAA CGT ATG GTA GGC TTT TGG ACG CAA   1512

 F   A   Q   S   G   N   P   N   N   E   E   I   P   G   M   A   N   L     522
TTT GCA CAA AGT GGT AAT CCA AAT AAT GAG GAA ATT CCT GGT ATG GCA AAC TTA   1566

 T   W   D   P   L   K   K   S   S   P   K   L   N   C   L   N   I   S     540
ACA TGG GAT CCA CTG AAG AAA AGT TCA CCG AAA TTA AAT TGT CTG AAT ATA AGT   1620

 D   D   L   K   L   I   E   W   P   E   L   A   K   A   K   V   W   A     558
GAT GAT TTG AAA CTA ATC GAA TGG CCG GAA TTG GCC AAA GCG AAG GTG TGG GCA   1674

 N   A   Y   D   A   H   K   E   L   L   Y                               570
AAT GCT TAC GAT GCG CAT AAA GAA TTA TTG TAT                              1707

AAAGGGGACAAAGGAGTGGTATAAAAAACACAAATATTGTCAAAAAACAATTCAATACAAAAGTAGTCATA
TCTGTGTAACTCAAAATGTGTATATATAGTATGTATTTTCTTAATTTATTTAAATACAATATATAATTTGT
TAATTATGCATTTGTTGTTTGTACATACTCGTATATTGTATTGGTTTATTAAAAAAAAATATTTTTTTACA
TATATATTTATATAGATTTGTGTATCAGGTAGTTAAATAGTAATCAATCTCATTAACCAAAATTCCACTTC
AAAAGCTTTATAAAACTATAAATTATGTACAAAATTTAAATAAATAAAACACAAAGGCTGCTTTCAACCAA
GCATGGAAAAAAAAAAAAAAAAAAAAAAAAA
```

Figure 1. The complete nucleotide sequence and the inferred amino acid sequence (single letter code) of the cDNA from the C strain of *C. capitata*. Catalytic triad is indicated by an (*) and the pentapeptide GESAG, acid turn (SDEG) and histidine loop (GVAHGDELS) motifs are underlined. The α-esterase motif (PLYLYRFDFDS) is in red.

4.2.3 Comparison of *αE7* cDNA sequences in the susceptible (C) and resistant (W) strains

A fragment of 1614 pb (positions from 59 to 1673) of cDNA, that comprises most of the coding region of CcαE7 was amplified by RT-PCR and direct sequencing from adult abdomens of 6 susceptible and 5 resistant individuals. When the cDNA sequences were compared, 29 nucleotides differences were observed, but only 6 of them resulted in changes in the amino acid at this position in the predicted protein (Table 4). Only one substitution Asn52Asp (in C. capitata) was found in homocigosis in all the individuals analyzed from the W strain. The substitutions Ala30The, Pro54Leu and Ser398Ala were always present in individuals from the W strain in homocigosis or heterocigosis and the other substitutions Val96Leu, Leu258Tyr, Phe410Leu and Asn460Ser were found in only some of the individuals analyzed from the W strain. None of them corresponded to the Gly137Asp and Trp251Ser/Leu substitutions.

Table 3. Amino acid substitutions found in the comparison of the predicted CcαE7 protein of different individuals from the C and the W strains of *C. capitata*.

Position[a]	C-1	C-2	C-3	C-4	C-5	C-6	W-1	W-2	W-3	W-4	W-5
30	A	A	A	A	A	A	A/T	T	T	A/T	A/T
52	N	N	N	N	N	N	D	D	D	D	D
54	P	P	P	P	P	P	P/L	L	L	P/L	P/L
96	V	V	V	V	V	V	V/L	V	V	V	V/L
258	L	L	L	L	L	L	L	Y	L/Y	L/Y	L/Y
398	S	S	S	S	S	S	S/A	A	S/A	A	A
410	F	F	F	F	F	F	F/L	F	F	F	F/L
460	N	N	N	N	N	N	N	N	N	N/S	N

[a] Position in *CcαE7* of *C. capitata*.

[b] The amino acid are indicated with 1-letter code.

4.2.4 Expression of CcαE7 in *C. capitata*

To investigate possible resistance associated to quantitative differences in esterase gene expression, RNA from single individuals of the susceptible C and the resistant W strains was examined by QPCR. A 1.5 fold increase in the expression of CcαE7 in the W strain with respect to the C strain was found that, however, was not significantly different ($p>0.05$, Mann-Whitney test).

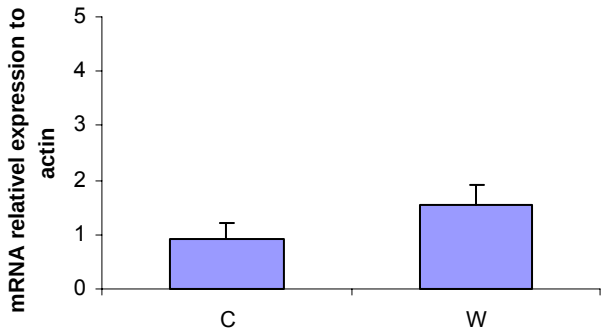

Figure 2. αE7 mRNA levels in 18 individuals from each strain of *C. capitata*, C and W. Data represented are the ratio of AChE to actin ± standard error.

4.3 Discussion

A modified AChE has been associated with malathion resistance in field populations of C. capitata (Chapter 3). However, metabolic resistance mediated by esterases may also be involved, since the esterase inhibitor DEF partially suppressed the resistance to malathion (Chapter 2). In the present study, we have found small differences in the specific activity against the esterase substrates MUH, MUB and MtB when the susceptible (C) and the resistant (W) strains were compared, and no differences for the rest of the substrates tested, including the naphthyl esters α-Na and β-Na. This results contrast with the pronounced increases in esterase activity reported for those species where resistance is due to over-expression mediated by gene amplification, as in Myzus persicae (Field and Devonshire, 1998; Field et al., 1999; Blackman et al., 1999) and mosquitoes (Gullemaud et al., 1997; Hemingway and Karunamaratne, 1998).

Cytochrome P450 and GST are other detoxification systems that have been associated with resistance to malathion (Weeling et al., 1974; Morton and Holwerda, 1985; Maitra et al., 2000; Taskin and Kence, 2004). The results obtained here do not show evidences that P450 or GST could be involved in the resistance to malathion in C. capitata, since no differences in specific activity were detected between the resistant W and the susceptible C strains. These biochemical assays support previous results from bioassays with synergists (Chapter 2), in which neither piperonyl butoxide nor dimethy maleate synergized malathion toxicity. However, further studies are needed,

since both P450 and GST belong to large superfamilies, and a single model substrate for general enzyme activity is not enough for drawing solid conclusions.

Point mutations in the M. domestica αE7 cDNA (Taskin and Kence, 2004; Taskin et al., 2004) and in its homolog E3 malathion carboxylesterase (MCE) from the L. cuprina (Campbell et al., 1998a) have been involved in specific resistance to malathion. In both cases, they showed reduced ability to hydrolyze aliphatic ester substrates, such as MtB, but acquired ability to hydrolyze OP substrates. We have found a significant reduction (1.6-fold) in the W strain in the esterase activity against MtB compared to the activity in the C strain. However, very low levels of in vitro metabolism of malathion were detected in both the C and W strains, which contrast with the increase in MCE activity reported for those species where this type of resistance has been confirmed (Whyard et al., 1994; Campbell et al., 1998a). Likewise, we found that the synergist TPP only slightly increased the toxicity of malathion in both C (2-fold) and W (3.2-fold) strains (Chapter 2), whereas completely restored the susceptibility to malathion when MCE is the main resistance mechanism (Hughes et al., 1984; Hemingway, 1985). Moreover, when the CcαE7 cDNA was sequenced, none of the mutations associated with resistance to OPs (Gly137Asp and Trp251Ser/Leu) (Newcomb et al., 1997; Claudianos et al., 1999) were found in individuals from the W strain. Thus, a hydrolytic mechanism mediated by MCE does not appear to be involved in the resistance to malathion in C. capitata. Over-expression of αE7 has also been implicated as a mechanism of resistance to OPs in H. irritants (Guerrero, 2000). However, the results obtained here do not indicate significant increases in the expression of CcαE7 in the W strain.

The predicted CcαE7 protein (Figure 1) was examined by alignment with other members of the carboxyl/cholinesterases multigene family [αE7 of higher Diptera: D. melanogaster; M. domestica, H. irritans and L. cuprina; AChE of M. domestica (Accession no. CAC39209) and; the juvenile hormone (JHE) of Manduca sexta (Accession no. AAG42021)]. It has 58%, 54%, 54% and 57% similarity to αE7 of D. melanogaster, L. cuprina, M. domestica and H. irritans respectively, and contains the three active motifs characteristic of carboxyl/cholinesterases multigene family (Oakeshott et al., 1993; Cygler et al., 1993): the catalytic triad which is composed by three no consecutive residues in the primary sequence, a nucleophile residue (Ser218 in C. capitata), an acid residue (Glu352) and His469; the residues around the catalytic triad, consisting of the pentapeptide Gly-Glu-Ser-Ala-Gly (residues 216-220), the acid turn (residues 349-352) and the histidine loop (residues 466-471); and the oxyanion

hole which is predicted to be formed by Gly136 Gly137 and Ala219 in C. capitata. Besides these motifs, CcαE7 has a distinctive PLYLYRFDFDS motif at residues 442-448 which is only conserved in α-esterases (Robin et al., 1996). No amino acid substitutions in the CcαE7 protein were found in the six individuals from the C strain. However all individuals from the W strain presented at least one substitution. The Asn52Asp change was the only present in all individuals analyzed from the W strain. It is located near the N-terminal of the predicted protein, and has not been reported before in resistant insects. All the other substitutions were in heterozygosis or in only some the individuals of the C strain. These substitutions may be involved in the reduction of the ali-esterase activity found in the individuals of the W strain, but do not appear to confer MCE activity.

From the results of this study, it appears that neither over-expression of detoxification enzymes nor a mutated CcαE7 that confer specific malathion resistance play an important role in the resistance of C. capitata to malathion. Nevertheless, the esterase inhibitor DEF partially suppressed the resistance to malathion (Chapter 2) and significant differences in activity were found for some esterase substrates. Whether these differences can be related with resistance of C. capitata to malathion remains to be determined.

5 Conclusions

1. Resistance to the organophosphorus insecticide malathion has been found, for the first time, in field populations of *Ceratitis capitata*. Differences in susceptibility can be related to the frequency of the treatments, being the populations from the Comunidad Valenciana, subjected to the greatest selection pressure, those that presented the highest levels of resistance.

2. A malathion resistant strain (W), derived from a field population from Castellón, showed less susceptible (9-fold) to another organophosphorus insecticide, fenthion, but not to the naturalite insecticide, spinosad, when compared with the susceptible laboratory strain (C).

3. Both, target site insensitivity and metabolic resistance appear to be involved in the loss of susceptibility to malathion.

4. The coding sequences of the acetylcholinesterase (AChE) and ali-esterase (αE7) have been obtained for the first time from *C. capitata*.

5. A point mutation Gly328Ala (*Torpedo* numbering) in AChE has been associated with resistance to malathion. Adult flies from the resistant W strain that present this mutation in homozygosis were less sensitive to inhibition by malaoxon suggesting that target site insensitivity allows them to survive to high concentrations of the insecticide.

6. The synergists triphenyl phosphate (TPP) and S,S,S,tributyl phosphorotrithioate (DEF) enhanced toxicity of malathion by 3.2- and 8.0-fold, respectively, indicating that esterases may play a role in resistance.

7. All individuals from the resistant W strain showed reduced ali-esterase activity, but they were not able to hydrolyze malathion and did not carry the mutations associated with resistance in other higher Diptera. Nevertheless, significant differences in activity were found for some esterase substrates. Whether these differences can be related to resistance of *C. capitata* to malathion remains to be determined.

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